Regulation of α2 Chimaerin and Associated Phosphorylation Pathways in Neuronal Signalling and Morphogenesis

By

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Abstract

The generation of neuronal dendrites and axons is a highly dynamic process. These extensive structural changes are regulated by molecules such as GTPases, as well as kinases/phosphatases, which provide cyclical control mechanisms. Rho family GTPases are regulated, in part, by GAPs such as neurone-enriched chimaerin, and act through downstream kinase effectors, including cyclin dependant kinase 5 (Cdk5).

α2 chimaerin is a Rac GAP and phorbol ester receptor, which also displays Cdc42/Rac effector functions. From this study it appears α2 chimaerin exists in an auto- inhibited state in the cytosol and that DAG/phorbol ester association, or selected mutations translocates α2 chimaerin to the membrane, leading to GAP activation. α2 chimaerin GAP activity was implicated in PMA induced neurite collapse of N1E-115 neuroblastoma cells. PMA also promoted the association of α2 chimaerin with target proteins Collapsin Response Mediator Protein-2 (CRMP-2) and p35/Cdk5, possibly as a result of a conformational change.

The Rac effector, Cdk5 is a proline directed, Ser/Thr kinase, whose activity contributes to neurite outgrowth, growth cone collapse and neuronal migration. Deregulation of Cdk5 in neurodegenerative diseases is implicated in pathological changes. α2-chimaerin GAP domain was shown to interact *in vivo* both with p35, the neuronal activator of Cdk5 and with Cdk5 itself.

CRMP-2 interacted *in vivo* with α2 chimaerin SH2 domain, for which tyrosine phosphorylation was not essential. The functional correlates of these protein interactions of α2 chimaerin were investigated *in vivo*. CRMP-2, which is hyperphosphorylated in Alzheimer's disease was identified as a novel Cdk5 substrate *in vitro* with CRMP-2 Ser^{522} identified as the target site. Phosphorylation of CRMP-2 Ser^{522}, potentially primes for GSK-3β phosphorylation of CRMP-2 Ser^{518} and Thr ^{514} which alters CRMP-2 conformation.

α2 chimaerin was not a Cdk5 substrate but may be tyrosine phosphorylated, which could potentially regulate its activity or associations in neuronal cells.

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Abbreviations

AB/AM	Antibiotic/antimycotic tissue culture additive
ABP	Actin binding protein
AD	Alzheimer's disease
ADF	Actin-depolymerising factor
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
Amp	Ampicilin
APP	Amyloid precursor protein
APS	Ammonium persulphate
ARHGAP	Rho GTPase activating protein
ARF	ADP ribosylation factor
Arp	Actin related proteins
ATP	Adenosine triphosphate
BCR	Breakpoint cluster region
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine monophosphate
Cdc	Cell division cycle
Cdk	Cyclin dependent kinase
CNS	Central nervous system
CRMP	Collapsin response mediator protein
DAG	Diacylglycerol
ddH_2O	Deionised purified water
DH	Dbl homology domain
DMEM	Dulbecco's modified eagles medium
DNA	Deoxyribonucleic acid
DTT	Dithiothretiol
E.coli	Escherichia coli
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix

EDTA	Ethylenediamine tetraacetic acid
EGF(R)	Epidermal growth factor (receptor)
ER	Endoplasmic reticulum
ERK	Extracellular-signal regulated kinase
EVH1	Ena/VASP homology domain
F-actin	Filamentous actin
FAK	Focal adhesion kinase
FCS	Foetal calf serum
FGF(R)	Fibroblast growth factor (receptor)
G-actin	monomeric actin
GAP	GTPase activating protein
GDI	Guanine nucleotide dissociation inhibitor
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
GIT	G-protein coupled receptor kinase interacting targets
GSK	Glycogen synthase kinase
GST	Glutathione-S-transferase
GTP	Guanosine triphosphate
HCl	Hydrochloric acid
IPTG	Isopropyl-thio--D-galactoside
IRS	Insulin receptor substrate
JNK	Jun N-terminal kinase-activated protein kinase
LPA	Lysophosphatidic acid
MAP	Microtubule associated protein
MAPK	Mitogen activated protein kinase
MDia	Mammalian diaphanous
MLC	Myosin light chain
MRCK	Myotonic Dystrophy kinase-related Cdc42-binding kinase
NF	Neurofilament
NFT	Neurofibrillary tangles
NGF	Nerve growth factor
NRTK	Non receptor tyrosine kinase
N-WASP	Neural-Wiskott Aldrich Syndrome Protein
OCRL	Occulocerebrorenal syndrome of Lowe

OPHN1	Oligophrenin
PAGE	Polyacrylamide gel electrophoresis
PAK	p21 activated kinase
PARG	PTPL1 associated Rho GAP
PBS	Phosphate buffered saline
PDGF	Platelet derived growth factor
PDK	Phosphoinositide dependent kinase
PH	Pleckstrin homology domain
PHF	Paired helical filament
PI	Phosphatidylinositol
PI3K	Phosphoinositide 3 phosphate lipid kinase
PIP	Phosphatidylinositol phosphate
PIP_2	Phosphatidylinositol biphosphate
PIP_3	Phosphatidylinositol triphosphate
PIX	Pak interacting exchange factor
PKA	cAMP dependent Protein Kinase
PKC	Protein kinase C
PKD	Protein kinase D
PKG	cGMP dependent protein kinase G
PLC	Phospholipase C
PLD	Phospholipase D
PMA	Phorbol myristate acetate
PMSF	Phenylmethyl-sulfonyl fluoride
PSGAP	PH and SH3 containing Rho GAP
PTB	Phosphotyrosine binding domain
PTP	Protein tyrosine phosphatase
PTK	Protein tyrosine kinase
PVDF	Polyvinylidene fluoride transfer membrane
Rac	Ras-related C3 botulinum toxin substrate
Rho	Ras homologous member A
ROK	RhoA kinase
RTK	Receptor tyrosine kinase
SAPK	Stress activated protein kinase
SDS	Sodium doedecyl sulphate

Ser	Serine residue
SH2	Src homology 2 domain
SH3	Src homology 3 domain
Sos	Son of sevenless
SRGAP	Slit Robo Rho GTPase activating protein
SRF	Serum Response Factor
START	Star related lipid transfer domain
Tiam	T-cell lymphoma invasion and metastisis
TEMED	N, N, N', N'-tetramethylethylenediamine
Thr	Threonine residue
Tyr	Tyrosine residue
Tween 20	Polyoxyethylene sorbitan monolaurate
WAVE	WASP family verprolin homologous protein
WW	tryptophan tryptophan domain

Chapter One

Introduction

Protein signalling molecules include enzymes, whose catalytic activities are tightly modulated, as well as multi-domain adaptor proteins that build and regulate protein complexes. Cell signalling is strictly regulated by a variety of mechanisms. The eukaryotic cell is highly compartmentalised with internal membranes and organelles, providing micro-environments where the local concentration of signalling molecules are highly regulated. Protein synthesis is tightly controlled at both the transcription and translational level, while degradation is equally finely regulated. Post-translational modification of proteins (e.g. phosphorylation, prenylation) and association with cofactors (e.g phosphoinositides and cAMP/cGMP) also serve to regulate activity, binding properties, subcellular localisation and degradation.

Cells sense and respond to the local environment via surface receptors, which upon ligand association, trigger an intracellular signalling cascade. Numerous cell signalling pathways converge on the Rho GTPase molecular switches to modulate the cytoskeleton.

1.A The Cytoskeleton

. The cytoskeleton is the cells interior scaffold, consisting of three types of protein filaments; (actin filaments, microtubules and intermediate filaments) which extend throughout the cytoplasm. It is a highly dynamic structure allowing morphological alterations in response to environmental stimuli. Morphological changes permit cell migration and neurite formation, but the cytoskeleton also provides and maintains the framework for the high level of organisation required by eukaryotic cells.

1.A.1 Microtubules

Microtubules are polymerised filaments of tubulin which is itself a heterodimer of α and β tubulin. Tubulin is found in most cell types but is most abundant in the vertebrate brain. Microtubules are cylindrical structures with a hollow core formed from a ring of 13 tubulin heterodimers. Microtubules are polar, with polymerisation more rapid at one end (plus end) compared with the other (minus) (Desai et al., 1997). The minus end is associated with a distinct microtubule organising centre (MTOC). *De novo* synthesis of microtubules is slow but polymerisation and depolymerisation are rapid events and microtubules frequently switch between phases of growth and depolymerisation. The energy for this dynamic instability comes from the hydrolysis of GTP-bound by β tubulin, which occurs after assembly. GTP hydrolysis weakens the bonds holding these structures together thus promoting disassembly. Mature microtubules are found modified by acetylation and detyrosination which is thought to promote microtubule associating protein (MAP) binding (Westermann et al., 2003). MAPs control microtubule stability and the drug *taxol* also serves to stabilise these dynamic structures. MAPs are extremely varied, but in the brain fall into two classes, high molecular weight proteins (>200kDa) e.g. MAP-1/MAP-2 and the Tau proteins (55-62kDa). Microtubules in axons are uniformly orientated with the plus ends pointing away from the cell body, whereas in dendrites the orientations are mixed. Certain forms of Tau are only found in axons, where MAP-2 is excluded. MAP-2 only resides in dendrites and the cell body. MAPs include microtubule motors e.g. kinesins and dyneins, which use the energy of ATP hydrolysis to move along these track structures powering organelle movement and mitosis/meiosis. These motor proteins move unidirectionally with dyneins generally controlling organelle transport towards the cell interior (towards minus end) and

kinesins governing transport of synaptic vesicles along axons towards nerve terminals (toward the plus end).

1.A.2 Actin Filaments

Actin is the most abundant eukaryotic cellular protein, constituting approximately 5% of total cell protein, with numerous isoforms (α, β γ) in higher eukaryotes. Actin filaments (F-Actin) are polymers of the 43kDa actin monomers (globular or G-Actin). These monomers, associated with ATP molecules, polymerise in a uniform orientation to form a tight helical structure, whereupon ATP is hydrolysed to ADP. Like microtubules, actin filaments are polar, polymerising faster at one end (plus end). Polymerisation requires both the availability of existing filament ends and actin monomers (Dos Remedios et al 2003).

Drugs which bind and stabilise/destabilise actin filaments have also become useful tools to study actin behaviour in cells. *Cytochalasins* are fungal products that bind the plus end of filaments preventing further polymerisation while the *phalloidins* are toxins isolated from the *Amantia* mushroom that bind and stabilise actin filaments.

1.A.2.1 Actin binding proteins

More than 162 actin binding proteins have been identified to date, many of which compete for the same loci on actin (Dos-Remedios et al., 2003) regulating actin filament assembly/disassembly.

The cell maintains a large pool of monomer ATP-actin, poised for rapid polymerisation. The small globular protein profilin (Ampe et al., 1988) is one of the most highly expressed cytoplasmic proteins and binds ATP-actin monomers (Safer et al., 1997). Profilin acts to maintain a pool of actin monomers, which would be rapidly

depleted if plus end filaments were readily available. It is the role of capping proteins to prevent this. Capping protein (Cap Z) and gelsolin are the most abundant capping proteins, which serve to inhibit the association and dissociation of actin monomers to plus end filaments. Uncapping of both CapZ and gelsolin has been demonstrated by association with the membrane phosphoinositides ($PI(4,5)P_2$) (Schafer et al., 1996). $PI(4,5)P_2$ also binds profilin and serves to dissociate profilin/actin complexes. .

Severing proteins cut existing filaments into smaller ones, generating additional plus ends, thus potentially promoting actin polymerisation. Gelsolin, which is stimulated by Ca^{2+}, acts to sever existing filaments but then caps the newly generated plus end filaments inhibiting elongation. ADF/cofilins (cosediments with F-actin) also sever actin filament but are thought to promote depolymerisation rather than assembly (Carlier et al., 1997). The short filaments generated from cofilin severing may be stabilised and cross-linked by the Arp2/3 complex.

1.A.2.2 Actin filament nucleation

Spontaneous actin filament nucleation is unfavourable due to the instability of actin dimers and trimers but accessory proteins serve to catalyse this process. A complex of seven proteins, termed the Arp2/3 complex (Machesky et al., 1994), is the most well characterised initiator of actin filament nucleation. As well as nucleation, the Arp2/3 catalyses the branching of actin filaments promoting filament assembly from the sides of existing filaments at a strict angle of 70^0 (Mullins et al., 1998). The Arp2/3 complex is intrinsically inactive requiring activation by nucleation promoting factors including the WASp/Scar proteins (Higgs et al., 2001).

Formins have also been shown to directly nucleate actin (Pruyne et al 2002) independently of the Arp2/3. The conserved forming homology domains (FH1 and

FH2) are both implicated in the control of actin assembly. The proline rich FH1 binds profilin (Wantanabe et al 1997) and SH3 and WW containing proteins including Src (Tominaga et al., 2000) while the FH2 domain controls actin nucleation *in vitro* (Pruyne et al 2002) and actin assembly in vivo (Evangelesta et al., 2002), through potential stabilisation of actin dimmers (Pring et al 2003).

1.A.2.3 Actin filament depolymerisation

The energy from ATP hydrolysis is used to drive actin polymerisation. Hydrolysis of ATP, by actin, upon polymerisation marks the filament for depolymerisation by the ADF/cofilin family members, which associate more readily with ADP-polymerised actin. Kinetically it is the dissociation of the generated phosphate that acts an effective timer for destruction. ADF/cofilin association also inhibits the rapid ATP exchange observed at the terminal plus end actin subunits, which serves to stabilise filaments. Phosphorylation of ADF/cofilins by LIM kinases, reduces their affinity for actin monomers and filaments (Blanchoin et al., 2000) thus stabilising actin filaments. Tropomyosin is a dimer of two identical α helixes which form a rigid rod shape capable of binding along the length of actin filaments. Tropomyosin association provides mechanical support and acts to inhibit ADF/cofilin association, thus strengthening and stabilising actin filaments (Bernstein et al., 1982).

Depolymerisation results in disassociation of ADF/cofilin-ADP-actin monomers. Profilin exchanges with ADF/cofilin and promotes ATP exchange (Blanchoin et al., 1998), replenishing the pool of ATP-actin monomers for polymerisation and freeing ADF/cofilin for another round of depolymerisation. Treadmilling is the continual flux of actin subunits from the minus end to the plus end.

1.A.2.4 Actin filament organisation

Dynamic surface extensions are common place in cells and are believed generated by forces produced from local actin polymerisation at the plasma membrane. Microspikes (5-10μM) and filopodia (< 50μM) are thin stiff protrusions of actin filaments organised into parallel bundles with their plus end pointing out. Fimbrin and villinis a widely distributed protein which organises the actin filaments into tight parallel bundles at the leading edge of cells (Bretscher et al., 1980, Bretscher et al 1979). α actinin and fascin is also found associated with filament bundles (Tilney et al 1995). Lamellipodia are thin sheet like extensions generated at the leading edge of migrating cells, consisting of a dense meshwork of actin filaments. At branching of angles of around 70^0 (Y-junctions) the Arp2/3 complex is localised (Svitkina et al 1999). Filamin A is a highly abundant actin crosslinking protein also found in lammelipodia at branches of higher angles (X-junctions) (Flanagan et al., 2002). Capping protein are also localised to lammelipodia such as gelsolin while cofilins localise to the rear of the lammelipodia (Svitkina et al., 1999). Stress fibres are loose bundles of actin filaments stretching across the cell, where they make contact with the plasma membrane forming an anchor structure (focal contact). Stress fibres are associated with myosin and the generation of contractile forces. Myosins are motor proteins capable of hydrolysing ATP upon association with actin filaments and this energy is used to move along the filaments in a minus to plus end orientation.

1.B Rho GTPases

The Rho GTPase family, which consist of at least 23 members, are key regulators of the actin cytoskeleton, controlling migration, vesicle trafficking, cell polarity and neurite outgrowth but have also been implicated in microtubule rearrangements, cell adhesion, transcription regulation and reactive oxygen species production(Etienne-Manneville et al., 2002).

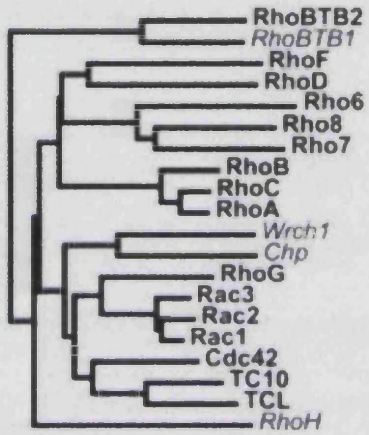


Fig.1.1 Rho GTPase family dendogram (taken from Snyder et al., 2003)

These 20kDa proteins are capable of binding and hydrolysing GTP. Two functional loop regions (switch I and switch II) surround the γ phosphate from the nucleotide and are conformationally altered upon GTP binding and hydrolysis (Milburn et al., 1990). GTPases are in effect molecular switches, "on" in their GTP bound forms and "off" when GDP bound (Bourne et al., 1990). When "on", they are capable of binding to and activating a diverse set of effector molecules, stimulating down stream signalling, which is self limited due to the protein's intrinsic GTPase activity. The cycle of activation is regulated by three classes of proteins: Guanine nucleotide exchange factors (GEFs) stimulate the exchange of GTP for GDP, turning the switch on, GTPase activating proteins (GAPs) stimulate the intrinsic GTPase activity, turning the switch off and guanine nucleotide dissociation inhibitors (GDIs) inhibit the dissociation of GDP but

also the hydrolysis of GTP therefore stabilising the switch. Rather than an on/off activity it is emerging cycling of the GTPases are crucial for dynamic functioning. The cycling of the Rho GTPases is tightly regulated with more than 60 GEFs and approximately 80 GAPs in the human genome.

These molecular switches must be spatially regulated as well as temporally. The C-terminal of most GTPases are post translational modified with the covalent attachment of a hydrophobic prenyl tail to a cysteine residue. The prenyl tail serves as a membrane anchor localising the GTPase activity to membranes (Hancock et al., 1989).

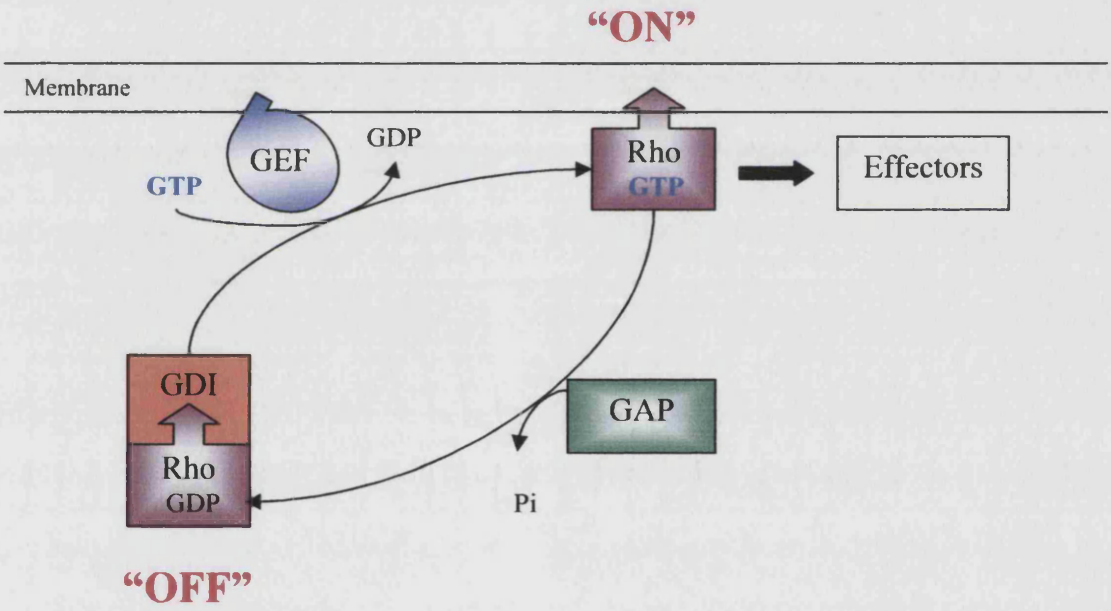


Fig 1.2 Rho GTPase cycle

1.B.1 Guanine nucleotide exchange factors (GEFs)

Members of the Dbl family of GEFs catalyse the release of the tightly bound GDP from the GTPase, stimulating GTP uptake in response to upstream signalling. Like the Dbl oncoproteins, many GEFs have been implicated in cancers e.g. T-cell lymphoma invasion and metastisis (Tiam1), Lbc and Lsc (Van Aelst 1997). GEFs act in a dual manner, to destabilise the GDP bound form and stabilise the non nucleotide bound

form, with the relative concentrations of cellular GTP/GDP driving the reaction. The DH (Dbl homology) domain is generally arranged in tandem with a 100 residue pleckstrin homology domain (PH), involved in membrane targeting, where the target prenylated Rho GTPases are located. In some cases the PH domain acts as negative regulator of the DH domain, which is relieved upon phosphoinositide association. As well as this catalytic 200-300 residue motif, a variety of other protein motifs connect GEFs to numerous signalling pathways.

Catalytic specificity varies amongst GEFs with Lbc and Lfc specific for Rho A, while Vav, is potentially active against RhoA, Rac 1 and Cdc42 (Olson et al., 1996). Trio is a Ser/Thr kinase with two distinct DH domains, displaying exchange activity towards Rac1 and RhoA respectively (Bellanger et al., 1998). Tyrosine phosphorylation of GEFs may serve as a regulatory mechanism, with Vav exchange activity upregulated by tyrosine phosphorylation (Crespo et al., 1997).

1.B.2 GTPase activating proteins (GAPs)

The Rho GAP family is defined by a conserved GAP domain of approximately 150 amino acids. Rho GAPs far out number their cellular substrates, implicating tight regulation of their activities and specific functioning. Their tertiary structure is very similar to that of Ras GAP (Bax et al., 1998), consisting of nine α helices and a conserved arginine finger (Gamblin et al., 1998). The GAP domain interacts with GTP binding core of the Rho GTPase (switch I and II and the P loop) inserting the positively charged arginine finger into the negatively charged active site. This serves to stabilise charges formed during the transition state of GTP hydrolysis, lowering the energy barrier, while at the same time positioning the catalytic glutamine residue of the GTPase

to coordinate the nucleophillic attacking water molecule. GAPs also serve to stabilise the switch regions, which is an essential feature of GAP activity (Fidyk et al., 2002).

Studies in general have only characterized Rho GAP activities against the most well defined Rho GTPase members (RhoA, Rac1 and Cdc42) *in vitro.* GAP activity varies from highly specialised to active against multiple members. ARHGAP6 is only active against RhoA (Prakash et al., 2000) while OPH1 is active against all three members (Billuart et al., 1998). CeGAP from *Caenorhabditis elegans* surprisingly acts on human Ras and Rab3A as well as several Rho family members (Chen et al., 1994). The presence of a Rho GAP domain in the regulatory subunit of PI3K, p85α and p85β, permits the association with and activation by Rac 1/Cdc42, although no GAP activity has been detected (Zheng et al., 1994, Bokoch et al., 1996). Similarly inositol polyphosphate 5-phosphatase GAP domain is devoid of activity (Jefferson et al., 1995). In some cases the Rho GAP domain may thus serve as a Rho GTPase interactive motif rather than a true catalytic GAP.

1.B.2.1 Rho GAP regulation

From their sequences Rho GAPs, in general, are multi domain proteins with a variety of protein and lipid binding domains, implicating complex regulatory mechanisms.

1..B.2.1.1 Lipid association

Rho GTPases are active on membranes and thus lipid association is a crucial requirement of many Rho GAPs. Cysteine rich domains (C1) have been found in several Rho GAPs including chimaerins (Ahmed et al., 1990), Gmip (Aresta et al., 2002), PARG1 (Saras et al., 1997) and MgcRacGAP (Toure et al., 1998). Lipid association

regulates chimaerin Rac GAP activity (Ahmed et al., 1993, Caloca et al., 2003) while the functional significance of the C1 domains in other GAPs remains to be determined.

Pleckstrin homology (PH) domains are also widely distributed amongst Rho GAPs including GRAF (Hilderbrand et al., 1996), Oligophrenin-1 (OPHN1) and PSGAP (Ren et al., 2001) where they have been presumed to be or shown to be required for correct localization. The Sec14 domain of p50RhoGAP is also believed to mediate phosphoinositide associations (Krugmann et al., 2002) while START (StAR-related lipid transfer) domains in p122RhoGAP (Homma et al., 1995) and other RhoGAP genes serve as an alternative lipid binding domain.

It was recently reported phospholipids can also switch the substrate preference of GAPs where phospholipids inhibited p190RhoGAP activity but stimulated Rac-GAP activity (Ligeti et al., 2004).

1.B.2.1.2 Protein interactions

As well as lipid binding domains, Rho GAPs have incorporated numerous protein binding motifs, linking the Rho GTPases with a variety of signalling pathways. The SH3 domain of GRAF mediates interactions with both Focal adhesion kinase (Hilderbrand et al., 1996) and the Rho effector kinase PKNβ (Shibata et al., 2001). Similarly PSGAP interacts with FAK and FAK related PYK2 (Ren et al., 2001). WW domains are also found in ARHGAP9 (Furukawa et al., 2001) and ARHGAP912 (Zhang et al., 2002).

Rho GAPs also posses proline rich regions themselves thus associating with a variety of SH3 containing protein. 3BP-1 associates with c-Abl (Cicchetti et al., 1992) while RICH-1 associates with CIP-4 SH3 domain (Richnau et al., 2001). PDZ (PSD-95/DlgA/ZO1 like) domain is another protein binding motif found in ARHGAP10

(Basseres et al., 2002). PARG1 has been shown to bind a PDZ domain in the protein tyrosine phosphatases PTPL1 (Saras et al., 1997) while Nadrin binds the PDZ of EBP50 (Reczek et al., 2001). Putative SH2 domains have also been found in Rho GAPs, linking them to tyrosine kinase signalling pathways. α2 chimaerin was found capable of binding phosphotyrosine residues, despite modification of the invariant N-terminal marking tryptophan (Hall et al., 2001).

Protein interactions potentially target Rho GAP activity or serve as an adaptor function. The first evidence of a protein association directly regulating GAP activity came from studies on CdGAP. The endocytic protein intersectin (Cdc42 GEF) was found to interact with CdGAP, via its SH3 domain, inhibiting GAP activity (Jenna et al., 2002). Alternatively Rho GAPs may regulate their associated proteins and p122RhoGAP was found to bind and activate PLCδ (Homma et al., 1995).

1.B.2.1.3 Phosphorylation

The association of numerous GAPs with kinases/phosphatases implicates phosphorylation as a further potential regulation point. Phosphorylation could serve to regulate Rho GAP activity, location or complex formation and tyrosine phosphorylation of numerous Rho GAPs have been observed. The Src kinase, Fyn phosphorylates p190RhoGAP (Wolf et al., 2001) and p250RhoGAP (Taniguchi et al., 2003, Moon et al., 2003) during oligodendrocyte maturation. p190RhoGAP is phosphorylated by Src kinases at residues close to the GAP domain (Roof et al., 1998) where upon tyrosine phosphorylation recruits p120RasGAP which stimulates p190RhoGAP activity. PSGAP also associates with both FAK and PKY2. Co-expression of PSGAP with PKY2, but not FAK, in cells leads to tyrosine phosphorylation and inhibition of Cdc42-GAP activity (Ren et al., 2001).

Ser/Thr phosphorylation of Rho GAPs has also been observed and found regulatory. PKC has been shown to phosphorylate p190RhoGAP inducing translocation to membrane ruffles (Brouns et al., 2000). GRAF was observed phosphorylated by Mitogen activated protein kinase (Taylor et al., 1998) and RICS (p250RhoGAP) phosphorylation by Ca^{2+}/Calmodulin dependent protein kinase II directly inhibited GAP activity (Okabe et al., 2003). Interestingly Ser phosphorylation of MgcRacGAP by Aurora B, during cytokinesis, was found to regulate substrate specificity *in vitro*, switching from Rac to Rho (Minoshima et al., 2003).

1.B.2.2 Rho GAP effector functions

The lack of GAP activity of some Rho GAP domains has suggested this domain may function as a Rho GTPase binding motif for possible effector function. GAPs could also conceivably promote dynamic GTPase signalling through increased cycling rates in cooperation with a GEF. Evidence of GAPs potentiating GTPase signalling do exist. The isolated GAP domain of α1 chimaerin serves to inhibit Rac stimulated lamellipodia formation. Full length protein however, cooperates with Rac1 and Cdc42 to induce lamellipodia and filopodia structures, requiring Rac binding but not GAP activity, suggestive of an effector function (Kozma et al., 1996). TCGAP translocates to the plasma membrane upon insulin stimulation and interacts with Cdc42 and TC10β (Chiang et al., 2003). GAP activity has only been demonstrated *in vitro* and is undetectable in cells implying possible effector function or masking of GAP activity *in vivo*.

A number of Rho GAP members also contain other catalytic domains for multiple functions including the kinase and Cdc42-GEF domains of BCR. ARAPS contain both Rho and Arf GAP domains and provide potential points of cross talk

between Rho and Arf GTPase signalling pathways (Miura et al., 2002). RICH1B contains a domain with homology to endophilins (Richnau et al., 2001), in which it functions as an LPA acyl transferase causing invagination of the plasma membrane (Schmidt et al., 1999). The role of this domain in RICH1B is thus highly intriguing and may implicate a function in vesicle formation. OCRL1 (oculocerebrorenal syndrome of Lowe) is a Rho GAP but also a PI(4,5)P_2 5 phosphatase, providing another crucial link between Rho GTPase and PI(4,5)P_2 signalling (Faucherre et al., 2003).

1.B.3 Guanine nucleotide dissociation inhibitors (GDIs)

Unlike GEFs and GAPs, the Rho GDI family is extremely small in number. GDIs serve to maintain an inactive pool of the Rho GTPases in the cytosol, through detachment of the GTPases from the membrane by burying their prenyl tail and inhibition of nucleotide exchange. The ubiquitously expressed Rho GDI was the first identified, binding to Rho A and B (Fukumoto et al., 1990) and since found to associate with Rac and Cdc42. Weak association with the GTP forms of these Rho GTPases has also been demonstrated, inhibiting GTP hydrolysis (Hart et al., 1992 and Nomanbhoy et al., 1996).

1.B.4 Alternative mechanisms of Rho GTPase regulation

As well as prenylation and interactions with regulatory proteins, it has been reported that a few Rho GTPases interact homophically, mediated by the C-terminal polybasic region (Zhang et al., 2001). This oligomerisation results in self stimulated GAP activity with Arg^{186} suggested to function as an arginine finger (Zhang et al., 1999). Rac1 in the oligomeric state remains responsive to GEFs but not to GAPs and potentiates PAK activation. Oligomer dissolution is promoted by GDI association.

Phosphorylation of Rho GTPases has also been observed. PKA phosphorylation of RhoA and Cdc42 increased Rho GDI binding (Forget et al., 2002) with consequent extraction of Rho-GTP from membranes (Lang et al., 1996). Ser^{188} phosphorylation of RhoA by PKA was also shown to inhibit association with its effector ROK (Dong et al., 1998). Phosphoinositide associations of Rac also regulate GDI binding (Chuang et al., 1993, Missy et al., 1998).

1.C The Rho GTPases and the cytoskeleton

The Rho GTPases exert their function through a diverse set of effector proteins, which bind the active GTP form. Over 60 effector proteins for Rho, Rac and Cdc42 alone have been identified, implicating Rho GTPases in the regulation of several signal transduction pathways (Etienne-Manneville et al., 2002).

1.C.1 Actin Dynamics

The first identified function of the Rho GTPases was actin cytoskeletal regulation. Microinjection of Rho A into fibroblasts cells resulted in stress fibre and associated focal adhesion formation (Ridley et al., 1992). Rac 1 promoted lamellipodia formation (Ridley et al., 1992), while Cdc42 was found responsible for filopodia induction (Kozma et al., 1995, Nobes et al., 1995). These three members (Rho A, Rac1 and Cdc42) are the most well characterised of the Rho GTPase family and have been implicated in extracellular receptor stimulated actin reorganisation. Lysophosphatidic acid (LPA) activates Rho (Ridley et al., 1992), Platlet derived Growth factor (PDGF) activates Rac (Ridley et al., 1992) and Bradykinin activates Cdc42 (Kozma et al., 1995).

Rho kinase (ROK) (Leung et al., 1995) was the first identified effector with direct links to the actin cytoskeleton (Leung et al., 1996). ROK in association with

Rho-GTP was found to phosphorylate and inhibit Myosin Light Chain (MLC) Phosphatase (Kimura et al., 1996) and phosphorylate MLC (Amano et al., 1996). Upregulation of MLC phosphorylation by ROK promotes myosin association with actin filaments leading to stress fibre formation and actin/myosin contraction. ROK was also found to phosphorylate and activate LIM kinase, which in turn, inactivates cofilin, thereby inhibiting actin depolymerisation (Ohashi et al., 2000). P140 mDiaphanous, another Rho effector, is a formin related protein, capable of binding profilin and stimulating actin polymerisation (Watanabe et al., 1997).

The first identified effectors of Rac1/Cdc42 were the Ser/Thr kinases, p65 PAKs (p21 activated kinase) (Manser et al., 1994). Rac1/Cdc42-GTP association induces a conformational change, relieving kinase autoinhibition, promoting autophosphorylation and phosphorylation of exogenous substrates (Chong et al., 2001). PAKs phosphorylate and inactivate MLC kinase (Sanders et al., 1999), thus down-regulating phosphorylation of MLC, affecting actin/myosin contraction. Phosphorylation and activation of LIM kinase by PAK has also been demonstrated (Edwards et al., 1999) which in turn alters cofilin activity. Another Cdc42 effector, myotonic dystrophy kinase-related Cdc42 binding protein kinase (MRCK) also phosphorylates MLC affecting actomyosin contractility (Leung et al., 1998).

The WASP family of Cdc42 effector proteins provided another crucial link between the Rho GTPases and the cytoskeleton (Rohatgi et al., 1999). Members of this family are capable of binding and activating the Arp2/3 complex, stimulating actin polymerisation (see 1.D.2.3). WASP proteins exist in an inactive conformation which is relieved upon GTP-Cdc42 and PI(4,5)P_2 association (Rohatgi et al., 2000), stimulating actin polymerisation at the plasma membrane. Rac has also been implicated in Arp2/3 activation by WAVE, via association through the adaptor IRSp53 (Miki et al., 2000).

IRSp53 has two distinct Rho GTPase binding sites for Rac 1 and Cdc42 and may provide another point of cross talk between the Rho GTPases. PIP5kinase is also a target of Rac, stimulating $PI(4,5)P_2$ production (Tolias et al., 1995). In platelets, this has a direct effect on actin dynamics leading to plus end uncapping and severing of actin filaments, stimulating actin polymerisation and lamellipodia formation.

1.C.2 Tubulin Dynamics

The microtubule and actin cytoskeleton are not isolated, but regulate each other. Thus Rho GTPases may modulate microtubule dynamics indirectly, through their effects on the actin cytoskeleton, but evidence also exists for a direct regulation. *In vitro* Rac 1 binds directly to tubulin in a GTP dependent manner (Best et al., 1996). Through the actions of PAK, Cdc42/Rac inactivate the microtubule destabilising protein, stathmin (Daub et al., 2001) while Cdc42 has been shown to regulate the orientation of the microtubule organising centre (MTOC) (Palazzo et al., 2001). The Rac/Cdc42 effector, IQGAP, serves to target microtubules to the leading edge via associations with the microtubule plus end targeted protein CLIP-170 (Fukata et al., 2002). The Rho effector mDiaphanous has also been shown to stabilise microtubules (Palazzo et al., 2001). Several GEFs including p190RhoGEF (van Horck et al., 2001) and Lfc (Glaven et al., 1999) have been localised to microtubules while the Rac GAP, α2 chimaerin, appears to bind tubulin via it's SH2 domain (Ferrari Thesis 1999). Collapsin response mediator protein (CRMP-2) binds tubulin heterodimers promoting microtubule polymerisation (Fukata et al., 2002) and is a substrate of ROK activity (Arimura et al., 2000). The Rac effector p35/Cdk5 also phosphorylates the microtubule binding proteins Tau and MAP-1B, regulating microtubule stabilisation (Baumann., et al 1993, Paglini., et al 1998).

Microtubule dynamics also regulate the Rho GTPases with polymerisation activating Rac (Waterman-Storer et al., 1999) and depolymerisation Rho (Ren et al., 1999). (GEF)-H1 is a Rho specific GEF which binds inactively to microtubules, but is released and activated upon microtubule depolymerisation (Krendel et al., 2002).

The Rho GTPase coordinate the cytoskelton to regulate dynamic biological processes such as cell migration, differentiation and vesicle trafficking, all of which are especially apparent in the nervous system.

1.D Central Nervous System (CNS) Development

The central nervous system (CNS) consists of highly specialised cells (neurones) and their supporting cells (glia). The billions of neurones of the human brain are interconnected, forming a complex network allowing for diverse signalling. Neurones are highly polarised cells adapted for receiving and transmitting signals. They have developed elongated process called axons and dendrites that extend over relatively long distances and connect to other neurones. Input signals are received by the dendrites, processed and the output transmitted through the axon. Signalling within the CNS consist of electrical impulses that flow across neurones, passing from one to the other via synaptic connections. Here neurotransmitters are released, which induces the firing of the connecting neurones, propagating the signal.

Neurones do not divide once differentiated and so are given a "birthday", defined as the time of final mitosis which generated the neurone from the neuronal precursor cell. Immature neurones then migrate from their place of birth guided by various factors. In vertebrates, neurones settle into six neuronal laminae within the forebrain. This program of neurogenesis occurs in an "inside-out" manner with early

generated neurones settling into the deepest layers and later neurones in the more superficial layers. Radial migration along glial fibres is the most prominent form of migration in the mammalian cortex. Once the neurone has reached its desired location it exits the radial glial track and differentiates. Differentiation occurs with the development of neurites, which elongate to form a single axon and multiple dendrites. The outgrowth of these neurite structures is tightly regulated, ensuring they extend to the correct locations. At the tips of these structures is the growth cone, which acts as a sensory structure, guiding and powering the movement. CNS development thus requires dramatic neuronal morphological adaptations to first migrate to their correct destinations and then extend out axons and dendrites.

1.D.1 Neuronal Migration

Cell Migration in general is regulated in part by the Rho GTPases (Raftopoulou et al., 2004). Through their various effectors, Rac/Cdc42, induce actin and microtubule polymerisation at the cell's leading edge. This, combined with the establishment of focal contacts, converts the forces of polymerisation into membrane extension. Coordinated contraction occurs at the rear of the cell through active myosin motors accompanied by efficient release of rear attachments with Rho/ROK signalling implicated, allowing forward movement. In the nervous system Cdk5 activity has also been implicated in neuronal migration.

1.D.1.1 Cyclin dependent kinase 5 (Cdk5)

This 33kDa proline directed Ser/Thr kinase is ubiquitously expressed in mammalian tissues but whose kinase activity is largely restricted to postmitotic neurones (Tsai et al., 1993, Lew et al., 1994). It is the restricted expression of its activators that determines

selectivity of activity. P35, a 35kDa protein, was the first identified and most well characterised activator of Cdk5. *In situ* hybridisation revealed p35 mRNA expression initiates as neurones exit the cell cycle and begin their journey of migration (Dellale et al., 1997). In mice, after birth, p35 mRNA levels rapidly decrease while Cdk5 levels increase, but a strong p35 signal remains in the adult brain associated with regions of high plasticity (pyriform cortex and pyramidal layer of the hippocampus). In brain lysates three separate pools of Cdk5 exist (Lee et al., 1996): isolated inactive monomeric Cdk5, relatively inactive Cdk5/p35 heterodimer in association with a large protein complex of 600kDa and an active p25/Cdk5 dimer. p25 is the C-terminal proteolytic cleavage product of p35 generated by the Ca^{2+} activated protease calpain (Lee et al., 2000). Cdk5 $^{-/-}$ mice display major defects in the hippocampus and cerebral and cerebellar cortices, reminiscent of reeler$^{-/-}$ mice, leading to perinatal death (Ohshima et al., 1996, Gilmore et al., 1998, Ohshima et al., 1999). These phenotypes have been mimicked by electroporation of a dominant negative Cdk5 into ventricular zone cells in the cerebral cortex (Kawauchi et al., 2003). P35$^{-/-}$ mice display severe cortical lamination defects of the cerebral cortex with a reversed layering of the cortical neurones in an "outside-in" pattern (Chae et al., 1997). This implies Cdk5 activity is essential for cortical neurones to migrate past their predecessors. The p35$^{-/-}$ mouse phenotypes appears to be only a subset of the Cdk5$^{-/-}$ phenoptypes with only mild hippocampal and cerebella abnormalities. P39 is another identified brain activator of Cdk5 with high similarity to p35 (Tang et al., 1995). p39$^{-/-}$ mice display no obvious brain developmental defects, implicating compensatory mechanisms between activators. Double mutant mice p35$^{-/-}$p39$^{-/-}$, exhibit identical phenotypes to Cdk5$^{-/-}$ mice, further implying p35 and p39 as the principal activators of Cdk5 (Ko et al., 2001), although p67 has also been identified as an activator (Shetty et al., 1995).

A preferred basic residue at the +3 position provides the consensus sequence S/TPX(K/H/R for Cdk5 phosphorylation. There are numerous identified substrates, including neuronal cytoskeletal elements. The lack of neuronal migration in p35/Cdk5 knockout mice could suggest there is an upregulation of neuronal adhesions. β-catenin was identified as a p35 interactor (Kwon et al., 2000) and p35/Cdk5 was shown to both phosphorylate β-catenin and interact with N-cadherin. Phosphorylation inhibits β-catenin-N-cadherin association, reducing cellular adhesion. The tyrosine kinase FAK is linked to focal adhesions in non neuronal cells and is crucial for migration (Parsons et al., 2000). Highest mammalian expression levels are found in the brain, where it is enriched in neurones in which typical focal adhesion structures are absent although analogous structures may be present (Contestabile et al., 2003). Cdk5 phosphorylation of FAK was shown to mediate microtubule reorganisation around the nucleus promoting nucleokinesis and neuronal migration (Xie et al., 2003). Cdk5 also mediates microtubule dynamics through phosphorylation of Tau and MAP2 proteins (Patrick et al., 1999, Berling et al., 1994).

1.D.2 Neurite outgrowth

During brain development, once the neuroblast has reached its destination, neuritogenesis occurs, which is believed to be mediated by the coordination of actin and microtubule cytoskeletons. Evidence indicates both cytoskeletons are dispensable for neuritogenesis as inhibition of microtubule polymerisation with nocodazole or of actin with cytochalasin B does not affect neurite formation (Smith et al., 1994, Marsh et al., 1984). Forces generated from the actin cytoskeleton were believed to break the neuronal sphere symmetry to induce a bud which develops to form an initial neurite.

Recent evidence implicates lamellipodia as the direct precursors to neurites (Dehmelt et al., 2003), which subsequently accumulate microtubules, forming the neurite shaft.

These minor neurites develop through the stabilisation of microtubules, redirection of membrane trafficking and interplay of gene transcriptional changes. One of the neurites develops into the axon while the remaining ones form dendrites inducing neuronal polarity, which is reproduced in culture on rat hippocampal cells (Barlett et al., 1984).

Rho GTPase signalling has been implicated in neurite outgrowth with opposing roles for Cdc42/Rac and Rho. Rho/ROK signalling induces neurite retraction in response to LPA in both N1E-115 neuroblastoma cells and PC12 cells (Jalink et al., 1994) while Rac/Cdc42 signalling is believed to promote outgrowth. Serum withdrawal of N1E-115 neuroblastoma cells results in neurite outgrowth in a Rac and Cdc42 dependent manner (Kozma et al., 1997). Over-expression of Cdc42/Rac effectors N-WASP (Edgeworth Thesis 2003), PAK5 (Dan et al., 2002) and IRSp53 (Govind et al., 2001) is also sufficient to promote neuritogenesis in N1E-115 neuroblastoma cells while mutants of N-WASP inhibit outgrowth in PC12 and hippocampal neurones (Banzai et al., 2000). Ras also promotes neurite outgrowth in N1E-115 cells, activating PI3K with down-stream Rac/Cdc42 activation (Sarner et al., 2000). Upregulators of Rac/Cdc42, including the GEFs Tiam (Leeuwen et al., 1997), STEF (Matsuo et al., 2002) and Trio (Bateman et al., 2000) also induce outgrowth, while the RhoGEF, ephexin induces retraction (Shamah et al., 2001). Exceptions to this general rule of Cdc42/Rac promoting outgrowth with Rho antagonism are exemplified by Sema 3A and Ephrin A2 induced growth cone collapse of neurones, which is Rac dependent (Jin and Strittmatter 1997, Jurney et al., 2002). RhoV14 expression in PC12 cells after NGF priming was

also found to increase neurite length while RhoN19 expression reduced neurite length (Sebok et al., 1999).

1.D.2.1 Rho GAPs

Evidence for the involvement of Rho GAPs in the nervous system came from the discovery of the Rho GAPs OPHN-1 and OCRL, which are associated with X-linked mental retardation (Billuart et al., 1998, Lin et al., 1997). Numerous Rho GAPs have been found to be brain specific or brain enriched including chimaerin (Ahmed., et al 1990), ARHGAP4 (Foletta et al., 2002) and Nadrin (Neuronal associated developmentally regulated protein), which regulates Ca^{2+} dependent exocytosis (Harada et al., 2000). SrGAPs (Slit Robo) are recently identified Rho GAPs involved in Cdc42 regulation, downstream of SLIT signalling (Wong et al., 2001).

P190RhoGAP is a well characterised Rho GAP required for axon outgrowth and guidance, mediating Src dependent adhesion. Fyn phosphorylates p190RhoGAP (Wolf et al., 2001) and p250RhoGAP (Taniguchi et al., 2003) during oligodendrocyte maturation and the transient over-expression of both p190RhoGAP and p250RhoGAP induces neurites in neuroblastoma cells (Brouns et al., 2001 and Moon et al., 2003).

α Chimaerins are phorbol ester receptors (Ahmed et al., 1990) with GTPase activity towards Rac (Diekeman et al., 1991, Manser et al., 1992). The C1 domain of α chimaerins is a Zn^{2+} dependent structure (Ahmed et al., 1991) involved in phorbol ester association and membrane translocation (Caloca et al., 2001), with lipid association regulating GAP activity *in vitro* (Ahmed et al., 1993). Two N-terminal splice variants of α chimaerin exist, α1 / α2 (Hall et al., 1990, Hall et al., 1993).

Fig.1.3 α Chimaerin members

α1 chimaerin mRNA is abundant in the adult brain associated with areas of learning and memory while α2 chimaerin is predominantly expressed in postmitotic neurones of the foetal brain (Hall et al., 2001). α2 chimaerin SH2 domain appears to alter subcellular distribution, adopting a more cytosolic location compared with the predominantly insoluble α1 chimaerin. The SH2 domain has been shown capable of binding phosphotyrosine residues, although specificity and *in vivo* receptors remain elusive.

α1 chimaerin, (Kozma et al., 1996) unexpectedly cooperates with Rac/Cdc42 to induce lamellipodia and filopodia in neuritic growth cones of N1E-115 cells, indicative of Rac/Cdc42 effector functions. Permanent expression of α2 chimaerin also induces neuritogenesis in N1E-115 neuroblastoma cells (Hall et al., 2001). No binding partners for α chimaerins have been identified for these possible adaptor functions or to anchor/target and regulate enzyme activity.

1.D.2.2 Collapsin response mediator protein (CRMP-2)

The neuronal specific CRMPs (1-5) are homologues of C.Elegans Unc33, mutants of which result in impairment of neural circuits and uncoordinated locomotion (Hedgecock et al., 1985). CRMP-2 is expressed immediately after neuronal birth and increases dramatically during cortical neurogenesis (Minturn et al., 1995). Expressed in neural cell bodies and neurites, it is also localised to filopodia and lamellipodia of growth cones (Minturn et al., 1995). Implicated in Sema3A growth cone collapse (Goshima et

al., 1995), CRMP-2 has also been found to induce axons in hippocampal neurones (Inagaki et al., 2001). Evidence suggests an involvement in tubulin dynamics (Gu et al., 2000 and Yuasa-Kawada et al., 2003) where CRMP-2 binds tubulin heterodimers to promote microtubule assembly (Fukata et al., 2002). CRMPs exist as both cytosolic and membranous bound forms with members localised to lipid rafts (Rosslenbroich et al., 2003), with the exception of CRMP-2, but CRMP-2 does associate with the plasma membrane oxidoreductase complex on synaptic vesicles and the synaptic plasma membrane (Buillard et al., 1997). Despite sequence similarity with dihydropyrimiinidase and heterotetramer formation (Deo et al., 2004), no enzyme activity has been detected (Wang et al., 1997). Only a few binding partners of CRMP-2 have been identified. Associations with Numb mediates endocytosis of proteins such as L1 (Nishimura et al., 2003) while the interaction with neuronal phospholipiase D_2 serves to inhibit PLD_2 enzyme activity (Lee et al., 2002).

CRMP-2 is a highly phosphorylatable protein and is found hyperphosphorylated associated with neurofibrillary tangles in tissue from Alzheimers disease patients (Gu et al., 2000). It is a target of Fps/Fes tyrosine phosphorylation downstream of Sema 3A signalling (Mitsui et al., 2002). CRMP-2 is also phosphorylated by ROK during LPA induced growth cone collapse (Aimura et al., 2000). Recently N-terminal splice variants of each CRMP have been identified (Leung et al., 2002 and Yuasa-Kawada et al., 2003). The new splice variant CRMP-1 A was found to form hetero-oligomers with CRMP-2 and associate with and inhibit ROK kinase activity (Leung et al., 2002). Recent work with non tagged protein also contradicted previous results. CRMP-2 reducedaxonal length and increased branching, which was abolished by co-expression of the novel, longer splice variant CRMP-2A (Yuasa-Kawada et al., 2003).

1.D.2.3 Cyclin dependent kinase 5 (Cdk5)

As well as regulation of neuronal migration, Cdk5 activity and has been implicated in neurite outgrowth through dominant negative and antisense studies (Nikolic et al., 1996, Xiong et al., 1997)). Cdk5 redistributes from the cell body toward axon growth cones in cultured neurones, during process formation, where it associates with the actin rich peripheral regions (Pigino et al., 1997, Paglini et al., 1998 and Nikolic et al., 1996). The short half life of p35 implies tight regulation of expression. Laminin engagement of integrins has been shown to upregulate p35 transcription and protein levels during neurite outgrowth (Paglini et al., 1998, Li et al., 2000). NGF induced outgrowth of PC12 cells also induces p35 expression through the ERK pathway (Harada et al., 2001). Studies imply Cdk5 activity does not simply promote outgrowth but acts as a key modulator of outgrowth (Pigino et al., 1997, Paglini et al., 1998 and Connel-Crowley et al., 2000).

Cdks are regulated by phosphorylation (see 1.A.2.1). Cdk activating kinase (CAKs) do not phosphorylate and activate Cdk5 (Qi et al., 1995) but Thr^{159} phosphorylation has been demonstrated by casein kinase I, which *in vitro* upregulates activity (Sharma et al., 1999). In contrast to Cdk2, phosphorylation of Cdk5 Tyr^{15} has been shown to increase kinase activity. C-Ableson (c-Abl) phosphorylates Tyr^{15} which is enhanced by the association of the adaptor protein Cables (Zuckerberg et al., 2000). Knockout of Cables reduces axon length while expression of active Abl increased axon length.

Cdk5 can influence cytoskeletal dynamics with the formation of filopodia and lamellipodia when p35 (Nikolic et al., 1998) and p39 (Humbert et al., 2000) are expressed in fibroblasts. Identified as a Rac effector, p35/Cdk5 also induces phosphorylation of PAK1 in a Rac dependent manner, which results in down regulation

of PAK1 kinase activity (Nikolic et al., 1998). Cdk5 also associates with the microtubule cytoskeleton (Sobue et al., 2000). Several microtubule binding proteins serve as substrates, including Tau (Baumann et al., 1993) and MAP1B (Paglini et al., 1998). Tau phosphorylation by Cdk5 reduces its affinity for microtubules (Patrick et al., 1999, Takahashi et al., 2003), inhibits microtubule assembly (Evans et al., 2000) and decreases microtubule nucleation ability (Wada et al., 1998). Influences of neurofilament dynamics are also observed with phosphorylation of the intermediate and heavy chains tail regions during outgrowth (Pant et al., 1997, Li et al., 2000, Grant et al., 2001), which can reduce axonal transport rates (Yabe et al., 2001, Ackerley et al., 2003).

Cdk5 activity is linked to axonal transport with kinase inhibition decreasing anterograde transport in giant squid axons (Ratner et al., 1998). Nudel is a recently identified substrate of Cdk5 that associates with the retrograde motor protein dynein (Niethammer et al., 2001). Phosphorylation of Nudel appears to regulate dynein function. P35/Cdk5 is also found to be active in Golgi membranes (Paglini et al., 2001), since its inhibition blocks the formation of membrane vesicles from the Golgi apparatus.

Increased sensitivity of $p35^{-/-}$ mice to seizures indicates Cdk5 might be involved in synaptic function (Chae et al., 1997). Cdk5 phosphorylates the presynaptic proteins synapsin 1 (Matsubara et al., 1996), Munc 18 (Shuang et al., 1998), amphiphysin 1 (Floyd et al., 2001) and Synaptojanin (Lee et al., 2004) *in vitro* all of which are involved in synaptic vesicle endocytosis. Phosphorylation of Munc 18 disassembles the Munc18/Syntaxin1 complex allowing Syntaxin1 to interact with vesicle SNARE proteins. Dynamin is a large GTPase essential for synaptic vesicle fission (Marks et al., 2001). Recently Dynamin 1 phosphorylation by Cdk5 (Tan et al., 2003) suggested an essential, *in vivo,* vesicle recycling role for Cdk5. However contradictory results

showed co-phosphorylation of dynamin 1 and amphiphysin 1 by Cdk5 serves to inhibit endocytosis (Tomizawa et al., 2003). Cdk5 influences synapse formation at the neuromuscular junction (Fu et al., 2001). Neuregulin increases Cdk5 expression and activity, leading to phosphorylation and endocytosis of the neuregulin receptor, ErbB, and downstream MAP/ERK signalling increases AchR expression.

Phosphorylation of DARP32 has implicated Cdk5 activity in dopamine signalling. PKA phosphorylates DARP32, which makes it a potent inhibitor of protein phosphatase 1 (PP1) and thus potentiates dopamine-induced phosphorylation of PKA substrates. Cdk5 activity has been shown to convert DARP-32 into a PKA inhibitor thus reducing dopamine signalling (Bibb et al., 1999). PKA and Cdk5 also both phosphorylate protein phosphatase inhibitor 1 with antagonistic consequences (Bibb et al., 2001). PKA phosphorylation converts protein phosphatase inhibitor 1 into an inhibitor of PP1, while Cdk5 phosphorylation reduces the substrate affinity for PKA. Cocaine dopaminergic signalling and p35/Cdk5 expression is found to be induced in rats administered cocaine (Bibb et al., 2001).

1.D.3 Axonal Guidance

In 1890 Ramon, J Cajal first observed the growth cone, at the tips of axons, which is now attributed the function of guiding and powering axon outgrowth. Growth cone motility and guidance is dependent on the dynamic actin and microtubule cytoskeletons. Actin dynamics is required for proper axon guidance although axon elongation can occur independent of actin assembly (Marsh et al., 1984 and Bentley et al., 1986). Engagement of the actin cytoskeleton with the ECM slows actin retrograde flow and utilises the forces of actin polymerisation to generate forward motion – "clutch

hypothesis" (Lin et al., 1995). Disruption of microtubule dynamics results in wandering growth cones with loss of persistent outgrowth (Tanaka et al., 1995)

Morphologically the growth cone consists of three regions, the peripheral region, the central region and the transition zone, consisting of actin bundles (actin arcs). The peripheral region consists of a radial array of actin bundled filopodia with an in between actin meshwork of lamellipodia. Microtubules are predominantly found within the central region although a subpopulation extend their plus ends into the F-actin rich peripheral region. Inhibition of actin assembly promotes microtubule advance into the peripheral region (Forscher et al., 1988) and it is now believed that microtubules grow along actin bundles and that actin retrograde flow exports microtubules out of the peripheral region (Schaefer et al., 2002). Microtubules that are exported often buckle and break within the transition zone and thus retrograde flow regulates microtubule turnover. Reorganisation of actin bundling induces growth cone steering and microtubule reorganisation (Zhou et al., 2002). Conversely promotion of microtubule polymerisation also appears to promote actin polymerisation inducing lamellipodia and growth cone turning (Buck et al., 2002). Microtubule and actin interactions are thus crucial for growth cone steering. The neuronal microtubule associated proteins Tau and MAP2 have been shown to crosslink microtubules with F-actin *in vitro* (Griffith et al., 1982) and phosphorylation has been shown to switch interactions of MAP2c from the microtubule to the actin cytoskeleton (Ozer et al., 2000).

As neurites elongate they must be guided to make the precise, required connections. Dynamic filopodia and lamellipodia at the leading edge of the growth cone are believed to sense the local environment. Guidance cues maybe attractive or repulsive; long or short range and many also influence neuroblast migration. As axons

migrate they must adapt and remain sensitive to the local guidance cue requiring both protein synthesis and degradation (Campbell et al., 2001, Ming et al., 2002).

Contact mediated guidance occurs through several families of cell surface adhesion molecules including N-CAM (neuronal adhesion molecule) and L1. Binding of ECM such as laminins and fibronectin to neuronal integrins promote outgrowth (McKenna et al., 1988). Signalling of membrane bound ephrins through Eph tyrosine kinase receptors, elicit both "forward" and "reverse" signalling pathways in receptor and ligand expressing cells.

Several families of secreted proteins have also been implicated in axon guidance (Netrins, Slits, neurotophins and Semaphorins). Whether these act as diffusible factors or factors bound to cell surface membranes and ECM is unclear:

1.D.3.1 Semaphorins

More than 30 Semaphorins (Sema) have been identified and divided into eight classes. These include transmembrane (clases 1, 4 and 6), GPI anchored (class 7) and secreted (2, 3 and V) proteins of which classes 1 and 2 are found in invertebrates, 3 to 7 in vertebrates and V in viruses.

Two families of transmembrane proteins serve as receptors for the semaphorins; the plexins (Tamagnone et al., 1999) and neuropilins (NPs). Association and activation of plexins is a characteristic of all members except the secreted class 3 Semas, which bind either NP1 or NP2 (Takahashi et al., 1999) and promote NP/Plexin association, relieving Plexin autoinhibition (Takashiti et al., 2001). Combinations of Plexins and NPs could generate specific responses to class 3 Semas with the possibility of additional molecules composing the Sema receptor complex. The cell adhesion molecule L1 associates with NP1, and L1 deficient axons fail to respond to Sema 3A (Castellin et al.,

2000). Similarly the kinase inactive drosophila receptor tyrosine kinase off track (Otk) associates with Plexs and functions downstream of semaphorin signalling (Windburg et al., 2001).

Plexins posses no known catalytic activity but do share minimal sequence similarity to Ras GAPs (Rohm et al., 2000). Plex B1 has been found to associate with RhoA and GTP-Rac1 (Vikis et al., 2000) and it has been proposed Plex B1 serves to sequester active Rac from downstream effector PAK (Vikis et al., 2002). Rac activity has however been shown essential for Sema3A collapse (Jin and Strittmatter 1997, Kuhn et al., 1999, Vastik et al., 1999) where it is required for endocytosis of the growth cone plasma membrane and reorganisation of F-actin following Sema 3A treatment but not for depolymerisation of F-actin (Jurney et al., 2002). PlexB has also been shown to activate Rho signalling through the RhoGEFs, PDZ-Rho-GEF (Driessens et al., 2002) and LARG (Leukeaemia associated RhoGEF) (Swiercz et al., 2002). Plex A1 binds Rnd1 causing collapse but association with RhoD inhibits collapse (Zanata et al., 2002).

Phosphorylation of cofilin by LIM kinase is essential for Sema3A collapse (Aizawa et al., 2001) which may be controlled by either a Rac or Rho pathway, although the significance of ROK activity in Sema 3A collapse is controversial (Arimura et al., 2000 and Dontchev et al., 2002). CRMP-2 was one of the first identified cytosolic proteins linking Sema collapse and the actin cytoskeleton, through antibody perturbation studies (Goshima et al., 1995). CRMP-2 lies downstream of ROK in LPA but not Sema 3A induced growth cone collapse (Aimura et al., 2000) but has been shown to switch Rho/Rac signalling in neuroblastoma cells (Hall et al., 2001). Evidence points to a role for CRMP-2 in dynamic regulation of the microtubule cytoskeleton (Gu et al., 2000 and Fukata et al., 2002). Fes/Fps tyrosine kinase was also

found to phosphorylate PlexA1, CRMP-2 and CRAM upon Sema 3A treatment (Mitsui et al., 2002).

Another tyrosine kinase implicated in Sema 3A collapse is Fyn (Sasaki et al., 2002) which localises to neuronal growth cones (Bixby et al., 1993). Fyn associates with and phosphorylates PlexA2 and Cdk5. Tyr^{15} phosphorylation of Cdk5 is essential for Sema 3A collapse and enhances kinase activity, which potentially inhibits PAK signalling (Nikolic et al., 1998) or may regulate tubulin dynamics through Tau/MAP phosphorylation (Sasaki et al., 2002). Sema 3A has also been shown to activate a pool of glycogen synthase kinase (GSK-3β) at the leading edge of growth cones (Eickholt et al., 2002) which is also involved in Tau phosphorylation (Mandelkow et al., 1992). Inhibitor studies reveal the potential involvement of PKG and PI3K activity in Sema 3A collapse (Dontchev et al., 2002).

1.D.3.2 Regulation of guidance cues

Guidance cues in general are bifunctional (attractant and repellent) and are classified into two groups depending on their regulation. Group 1 (BDNF, netrin1, acetylcholine, MAG) response is abolished by depleting extracellular Ca^{2+} and regulated by levels of cAMP/activity of PKA (Song et al., 1997). Decreasing cAMP converts netrin-1, NGF, BDNF attraction into repulsion (Song et al., 1999) while raising levels results in attraction (Ming et al., 1997). Group 2 guidance cues, including Sema 3A and NT-3, are regulated by levels of cGMP/activity of PKG (Song et al., 1998) and inhibition of cGMP signalling converts NT-3 attraction into repulsion.

Guidance cue signals interact and growth cones raised in NGF/BDNF become resistant to Sema 3A treatment where PKA but not PI3K activity is involved (Dontchev et al., 2002). Cross desensitisation experiments also provide evidence of common

signalling pathways by guidance cues (Ming et al., 1999) with group 1 members signalling through PLCγ and PI3K dependent pathways.

1.D.4 Neurodegenerative Diseases

Errors in signal transduction pathways can result in degenerative diseases. The presence of neurofibrillary tangles (NFTs) is a pathological hallmark of several neurodegenerative diseases including Alzheimers Disease, Down's Syndrome, progressive supranuclear palsy and Parkinson's disease. NFT compose primarily of paired helical filaments of hyperphosphorylated Tau (PHF-Tau) with 21 identified phosphorylation sites, ten of which are Ser/Thr-Pro motifs. CRMP-2 is also found hyperphosphorylated associated with NFT in AD although the responsible kinases are unknown (Gu et al., 2000).

Relatively few Tau kinases exist *in situ* including PKA, microtubule affinity regulating kinase, GSKβ and Cdk5. Cdk5 was shown to associate with NFT *in vivo* (Pei et al., 1998) and phosphorylate Tau on five sites in PHF-Tau (Paudel et al., 1993). Tau does not appear to be a physiological substrate of p35/Cdk5 but is of the more active p25/Cdk5. The N-terminus of p35 contains a myristolation signal, required for correct membrane localisation at the cell periphery (Patrick et al., 1999); as well as a Cdk5 phosphorylation site that initiates ubiquitin mediated degradation, which results in the short p35 half life of 20-30 min (Patrick et al., 1998). Both these signals are lost upon calpain cleavage of p35, resulting in sustained mis-localisaed Cdk5 activity. Highly phosphorylated p35 is found in the foetal brain where Cdk5 phosphorylation of p35 leads to rapid degradation which serves to protect from calpain cleavage (Kerokoski et al., 2002, Saito et al., 2003). In the adult brain p35 is susceptible to calpain cleavage and p25 accumulation is noted in Alzheimer's disease and amyotrophic lateral sclerosis

(ALS) where it is implicated in pathological conditions (Patrick et al., 1999). Transgenic mice over-expressing p25 display hyperphosphorylated Tau and neurofilament, as well as cytoskeletal disruptions associated with AD (Ahalijanian et al., 2000, Noble et al., 2003). Amyloid plaques are another pathalogical hallmark of AD with extracellular deposits of the fibrillogenic amyloid-β (Aβ) peptides derived from the amyloid precursor protein (APP). Treating primary neurones with Aβ peptides induces calpain activation (Nakagawa et al., 2000) and cleavage of p35 to p25 (Lee et al., 2000) with subsequent hyperphosphorylation of Tau (Busciglio et al., 1995), while inhibition of Cdk5 diminishes Aβ neurtoxicity (Alvarez et al., 1999). Cdk5 may thus link plaque deposition and NFT formation, although this model is disputed (Kerokoski et al., 2002, Tandon et al., 2003, Hallows et al., 2003). Cdk5 phosphorylates and activates p53 *in vitro* (Zhang et al., 2002) but conversely has also been implicated in neuronal survival, inhibiting JNK3 mediated apoptosis (Li et al., 2002) and activating PI3K/Akt signalling in a neuregulin dependent manner (Li et al., 2003).

The ubiquitous, constituitively active GSK-β, is brain enriched (Woodgett et al., 1990) and inactivated by Ser^{9} phosphorylation. GSK3β is capable of phosphorylating Ser/Thr-Pro motifs although in most cases the substrate must be previously phosphorylated at a site 4 residues C-terminal of the target site, which primes for GSK3 phosphorylation (S/T XXXpS/T) (Harwood et al., 2001). These sites are often found in proline rich regions of the substrate. GSK3β was found to co-purify with Tau and microtubules (Imahori et al., 1997). GSK3β phosphorylates Tau at both primed and unprimed sites (Ishiguro et al., 1993) and p25/Cdk5 activity has been shown stimulatory for GSK3β mediated phosphorylation of Tau at specific sites (Thr^{231}), reducing microtubule affinity (Cho et al., 2003). Aβ peptides activate GSK-3β in cell culture (Takashima et al., 1996) leading to Tau hyperphosphorylation (Takashima et al., 1998).

Activation of GSK3β contributes to neuronal apoptosis which PI3K/Akt phosphorylation of Ser^9 serves to inhibit (Hetman et al., 2000, Cross et al., 1998). GSK3β and Cdk5 activity are also implicated in APP protein phosphorylation, enhancing processing (Phiel et al., 2003, Liu et al., 2003).

1.E Aims

It was hypothesised that α2 chimaerin could play an important role in axonal outgrowth as a mediator of negative guidance cues via Rac GAP function, which may be regulated by protein/lipid interactions and phosphorylation. Having identified CRMP-2 and p35 as potential *in vitro* binding partners of α2 chimaerin the aims of this research were to:

1. Further characterise *in vivo* interactions of α2 chimaerin using pull down assays.

2. Establish whether Cdk5 phosphorylates α2-chimaerin or its novel target proteins *in vitro* and whether cdk5 phosphorylation affects interactions between these proteins.

3. Establish whether interactions of α2 chimaerin can regulate GAP activity *in vivo.*

4. Investigate the morphological effects of α2 chimaerin over-expression in the N1E-115 neuroblastoma cell model and the consequences of protein interactions.

This research could potentially shed some light on the signal transduction pathways governing neurite outgrowth/collapse during neuronal development

Chapter Two

Materials & Methods

2.A Materials

2.A.1 Laboratory Reagents

General Laboratory reagents were obtained from Sigma or BDH. PBS tablets were from Oxoid and water was purified in the lab using Elga Option 4 purification System (Deionised and purified by reverse osmosis before filtered and UV sterilised). Amersham supplied radioactive nucleotides

2.A.2 DNA Manipulation Reagents

Epicurian Coli XL1- Blue and BL-21 competent cells were obtained from Stratagene. DNA restriction enzymes were purchased from Boehringer Mannheim, Gibco-BRL and NEB and DNA modifying enzymes from Promega and Stratagene. The Wizard DNA purification mini-prep system from Promega and Qiagen's midi preparative kits were used. Agarose, HaeIII-digested ϕX174 and HindIII-digested λ7 DNA markers were purchased from Gibco-BRL and ampicillin was obtained from Sigma. Stratagene site directed mutagenesis kit was utilised

2.A.3 Tissue Culture Reagents

Dulbecco's Modified Eagle Medium (DMEM), foetal calf serum, antibiotic/antimycotic solution and lipofectamine were all obtained from Gibco-BRL. Trypsin and poly-L-lysine were purchased from Sigma

2.A.4 Protein Work Reagents

Commasie Brilliant Blue-R protein stain, glutathione agarose and Thrombin were from Sigma. Enhanced chemiluminescent (ECL) reagent and Hyperfilm were from Amersham as was ATP. ICN supplied polyvinyl difluoride (PVDF) membranes. Amicon protein concentrators were used as was Medicell's dialysis tubing. Biorad supplied TEMED and protein assay reagent to quantitate protein concentration. Acrylamide/bis acrylamide was purchased from Scotlab and NEB's prestained protein markers were used. Recombinant p25/Cdk5 was a gift from Dr R. Qi, while recombinant GSK3β and purified p55 Fyn was purchased from Upstate cell signalling solutions.

2.A.5 Antibodies

The following antibodies were used. Rabbit polyclonal α2chimaerin lab antibody (Hall., et al 2001) and mouse monoclonal 3F4 (Gu., et al 2000). M2-FLAG-AC, Rabbit polyclonal FLAG and β Tubulin were obtained from Sigma. Rabbit polyclonal Cdk5 (C-8), Cdk5-AC, p35 (C-19) and GST (Z-5) were obtained from Santa Cruz Biotechnology. Other antibodies used include mouse monoclonal HA (Babco), mouse monoclonal GFP (Clonetech). Transduction Lab mouse monoclonal Rac (R56220) and phosphotyrosine-HRP (RC20) were also used, as was mouse monoclonal P-Thr-Pro-101 (Cell Signalling Technology).

Donkey anti mouse or rabbit conjugated to FITC, TRITC or Cy5 (Jackson Immunochemicals) were used for cell staining along with TRITC conjugated Phalloidin (Sigma). DAKO's HRP conjugated pig anti rabbit, rabbit anti mouse and mouse anti rabbit were used for western blotting.

2.A.6 cDNA Constructs

Several cDNA constructs were used in this study. CMV2FLAG-p35, pXJ40HA-Cdk5, pXJ40HA-Cdk5N144, pcDNA3.1p35, pCl-neop35 were a generous gift from Dr Robert Qi (Dept Biochemistry, Hong Kong University of Science and Technology) as was HA-GSK3β from Dr Eikaholt (Molecular Neurobiology Group, Kings College London). pXJ40 HA/FLAG-CRMP-2/α2 Chimaerin constructs as well as pGEXGST-α2chimaerin were obtained from Dr Clinton Monfries, while pXJ40GST-CRMP-2 constructs were from Mathew Brown. GFP/HA–α2 Chimaerin SH2 mutants were as previously described (Hall et al 2001)

2.A.7 Oligomer Synthesis and cDNA Sequencing

Oligomers were synthesised by Genosys, Sigma. cDNA constructs sequenced by Cytomyx.

2.B Methods

2.B.1 DNA Manipulation

2.B.1.1 Competent bacterial transformation

20ul *Epicurian Coli* XL1- Blue competent cells (Stratagene) were thawed on ice and incubated with β-mercaptoethanol (25mM) for 10min. Approximately 100ng of the relevant DNA was added and the cells were incubated on ice for a further 30min. Cells were heat shocked at 42^0C for 45secs before incubating on ice for 2 min. Cells were allowed to grow at 37^0C in 1 ml L-Broth (2% (w/v) tryptone, 1% (w/v) yeast extract, 2% (w/v) NaCl) with vigorous shaking for 1hr before plating out on the relevant antibiotic containing (100μg/ml

ampicillin) agar plates (2% (w/v) tryptone, 1% (w/v) yeast extract, 2% (w/v) NaCl, 1.5% agar). Plates were placed inverted in a 37°C incubator for 16 hours to allow colonies to grow.

5ml antibiotic containing L-Broth were innoculated with a single colony, picked using a sterile toothpick. Cultures were grown for 16hrs in a 37°C incubator with vigorous shaking.

2.B.1.2 Plasmid DNA mini preparations

Promega Wizard Plus Miniprep DNA Purification System was utilised for small scale DNA preparations. 5ml cultures of the relevant bacterial cells were pelleted by centrifugation at 3000g for 5 min. The resulting pellet was resuspended in 1.5ml PBS and transferred to a 1.5ml microfuge tube, where the cells were repelleted. The cell pellet was thoroughly resuspended in 250µl cell resuspension solution (50mM Tris-Cl, pH8; 10mM EDTA, 100µg/ml RNase A). 250µl cell lysis buffer (200mM NaOH, 1% SDS) was added, mixed by inversion and incubated at room temperature for 5min. 10µl alkaline protease solution was added, mixed by inversion and incubated at room temperature for a further 5 minutes. Finally 350µl of neutralising buffer (3m potassium acetate, pH5.5) was added and mixed by inversion. After removal of cell debris by centrifugation at 18,000g for 10 minutes, the lysates were transferred to mini prep columns, which were placed into 2ml collection tubes. The columns were centrifuged at 18,000g for 1 minute and the flow through was discarded. Columns were washed twice with 250µl of column wash solution (1M NaCl, 50mM MOPS, pH7; 15% isopropanol) which was passed through the columns by centrifugation at 18,000g for 2 minutes at room temperature. Columns were transfered to clean microfuge tube tubes

and the plasmid DNA was eluted by the addition of 100µl of nuclease free water onto the mini prep columns, followed by centrifugation at 18,000g for 1 minute.

2.B.1.3 Plasmid DNA midi preparations

For greater yields of plasmid DNA, Qiagen's hispeed midi plasmid purification system was used. 250ml cultures of the relevant transformed XL-1 Blue were grown up in L-Broth, containing 50µg/ml ampicillin, with vigourous shaking at 37°C. Bacterial cells were pelleted by centrifugation at 3,000g for 15 minutes at 4°C in a Beckman J6-HC centrifuge. The pellets were washed by resuspending in 30ml PBS and repelleting by centrifugation. Bacterial pellets were thoroughly resuspended in 6ml buffer P1 (50mM Tris-Cl, pH8; 10mM EDTA, 100µg/ml RNase A) by vigorous pipetting. 6ml buffer P2 (200mM NaOH, 1% SDS) was added, mixed by inversion and incubated at room temperature for 5 minutes. 10ml of chilled buffer P3 (3M potassium acetate, pH5.5) was added to the lysed cells to neutralise the acid. Samples were mixed immediately by inversion and the lysate was poured into the barrel of a QIAfilter cartridge and incubated at room temperature for 10 minutes. A precipitate containing the genomic DNA, proteins and detergent forms and floats to the top of the solution in the cartridge. During this incuabtion period a HiSpeed Midi tip was equilibrated by the application of 4ml buffer QBT (750mM NaCl, 50mM MOPS, pH7; 15% isopropanol, 0.15% Triton X-100) which was allowed to pass through the tip by gravity.

Using a plunger, the cell lysate was filtered through the QIAfilter directly into the equilibrated tip, where it was allowed to enter the resin by gravity. The tip was washed with 20ml of buffer QC (1M NaCl, 50mM MOPS, pH 7; 15% isopropanol) and the DNA eluted with 5ml of buffer QF (1.25M NaCl, 50mM Tris, Tris-Cl, pH8.5; 15% isopropanol). The

eluted DNA was precipitated by the addition and incubation of 3.5ml (0.7 volumes) isopropanol (5min room temperature). The precipitated DNA was poured into a 20ml syringe and forced through a QIAprecipitator. Air was then forced through the precipitator using the empty syringe and finally the plasmid DNA was eluted by passing 1ml of TE buffer (10mM Tris-Cl, pH 8; 1mM EDTA) through the precipitator from a 5ml syringe. The elutant was reapplied to the 5ml syringe and passed through the precipitator again, to ensure maximum recovery of the plasmid DNA.

2.B.1.4 Phenol/Chloroform extraction

1.5ml phase light tubes (Sigma) containing an organic solvent penetratable gel were prepared by centrifuging at 16,000g for 2 minutes. DNA samples were placed into the prepared tubes with an equal volume of phenol. Samples were inverted several times until a white emulsion was formed and then centrifuging at 16,000g for 2 minutes. The gel in the phase light tubes formed an interface between the aqueous DNA containing layer and the phenol/RNA and protein containing layer. The upper aqueous DNA containing layer was removed and placed into fresh prepared phase light tubes with an equal volume of water saturated chloroform. Chloroform clean up was carried out as per phenol. The upper aqueous DNA containing layer was removed and the DNA was recovered by ethanol precipitation.

2.B.1.5 Ethanol precipitation

DNA samples were adjusted to pH5.2 with addition of 0.1 volumes of 3M sodium acetate pH 5.2 and two volumes of ice cold absolute ethanol before incubating at -20^0C for 20min. Samples were centrifuged at 18,000g for 5 minutes to pellet the DNA. The supernatant was removed by pippeting and vacuum desiccating. DNA was finally dissolved in TE buffer (10mM Tris-Cl, pH 8; 1mM EDTA).

2.B.1.6 Quantitation of DNA

Quantity and quality of DNA preps were evaluated by measuring the optical density at 260nm and 280nm wavelengths, using a deuterium lamp spectrophotometer. 5µl of purified plasmid DNA was diluted in 995µl of TE buffer. The optical density of each sample was measured in an UV cuvette at wavelengths of 260nm and 280nm. Since 1mg DNA gives an O.D of 50 at 260nm the concentration of DNA was evaluated using the following formula:

[DNA] µg/µl = 50 x (Dilution Factor) x (1/1000) x A_{260nm}

2.B.1.7 Restriction digestion of DNA

Various endonucleases were used to digest cDNA plasmid, for analysis. Restriction digests were typically carried out in 20µl reaction volumes, using the buffer of the relevant salt concentration provided by the manufacturer. Units of enzyme used in the reaction were determined by the amount of DNA present but "star activity" (non specific cutting) which results from glycerol present in the enzyme samples was avoided (>5% v/v contributes to star activity). Digests were carried out at 37°C for 1-2 hours. Reactions were quenched by heating to 65°C for 20 minutes

2.B.1.8 Agarose gel electrophoresis

10μl of linearised DNA was mixed with 2μl of 6 x DNA loading buffer (0.25%bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol in water) and loaded on a 1% agarose gel. 5μl of Hae III digested ϕX174 DNA (23.13kb to 125bp, 0.6μg/μl 6 x loading buffer) and Hind III digested λ DNA (1353bp to 72bp, 0.6μg/μl 6 x loading buffer) were also loaded and electrophoresed at a constant voltage of 140V for 45 minutes.

2.B.1.9 Visualisation of DNA with Ethidium Bromide

To visualise DNA bands, the gel was removed from the tank and soaked in distilled water containing 40μg/ml of ethidium bromide, for 15 minutes. Ethidium bromide is a DNA binding agent, which fluoresces when excited with ultra violet (UV) light (312nm wavelength). Thus after soaking in EtBr, DNA bands could be visualised by placing the gel on a UV transluminator and the data was recorded on polaroid film.

2.B.1.10 Site directed mutagenesis

To introduce point mutations in CRMP-2, Stratagene's Quickchange site directed mutagenesis kit was used on the FLAG-CRMP-2 pXJ40 plasmid. The procedure uses two synthetic oligonucleotide primers containing the desired mutation and *Pfu* Turbo DNA polymerase, to extend the primers by thermocycling. A mutated plasmid containing staggered nicks is thus generated and treatment with Dpn 1 endonuclease (target sequence: 5' Gm^6ATC-3') digests the parental methylated DNA template. The vector DNA is then transformed into XL10-Gold ultra competent cells.

Mutated primers design

CRMP-2 T509A
5'-GAG GTG TCT GTG GCG CCC AAG ACG GTC-3'
3'-CTC CAC AGA CAC CGC GGG TTC TGC CAG- 5'

CRMP-2 T514A
5'- CCC AAG ACG GTC GCT CCG GCC TCA TC-3'
3'- GGG TTC TGC CAG CGA GGC CGG AGT AG- 5'

CRMP-2 S518A
5'- C ACT CCG GCC TCA GCA GCT AAG ACA TCC-3'
3'- G TGA GGC CGG AGT CGT CGA TTC TGT AGG- 5'

CRMP-2 S522A
5'- CA GCT AAG ACA GCC CCT GCC AAG CAG-3'
3'- GT CGA TTC TGT CGG GGA CGG TTC GTC-5'

CRMP-2 T514E
5'- G ACG CCC AAG ACG GTC GAG CCG GCC TCA TCA GCT AAG-3'
3'- C TGC GGG TTC TGC CAG CTC GGC CGG AGT AGT CGA TTC- 5'

CRMP-2 S518E
5'- G ACG GTC ACT CCG GCC TCA GAG GCT AAG ACA TCC CCT GCC-3'
3'- C TGC CAG TGA GGC CGG AGT CTC CGA TTC TGT AGG GGA CGG-5'

CRMP-2 S522E
5'-GCC TCA TCA GCT AAG ACA GAG CCT GCC AAG CAG CAG GCG-'3
3'-CGG AGT AGT CGA TTC TGT CTC GGA CGG TTC GTC GTC CGC-5'

The PCR reaction was set up as follows:

5µl 10x reaction buffer

10ng of ds DNA template (FLAG-CRMP-2)

125ng mutant primers(5'→3' + 3'→5')

1µl dNTP mix

3µl Quik solution

ddH20 to final reaction volume of 50µl

1µl Pfu Turbo DNA polymerase (2.5 U/µl)

Tubes were placed into a Perkin Elmer PCR machine for the following cycles:

95°C for 30sec to denature template DNA.

95°C for 30 seconds to denature template DNA in later amplification cycles

X°C for 1 minute to allow annealing of mutated oligomers to template DNA

68°C for 13 min to allow extension of oligomers

This part of the cycle was repeated 16 times in order to amplify the required section of the template DNA.

68°C for 7 minutes to allow final extensions of oligomers

The value of X is based upon the Tm value of the oligonucleotides used in the reaction.

Tm = 81.5 + 0.41(%GC) –675/N -% mismatch

where N is primer length

1µl of Dpn 1 restriction enzyme (10U/µl) was added directly to each reaction and incubated for 1hr at 37^{0}C. XL-1 Blue competent cells were transformed and plated out as described.

2.B.1.11 Identification of positive mutant clones

To identify positive mutant clones one of the mutated oligos used in the clone syntheses was labelled and used as a probe to screen the colonies. N-Hybond filter lifts of the plate were taken using ink stabs to determine orientation. The master plate was allowed to recover again at 37^{0}C for 4hrs while the filter lifts were placed back inverted on a fresh agar plate and allowed to grow up at 37^{0}C for 2hrs. Filters were chilled at 4^{0}C for 30min before placing colony side up on presoaked 3mm Whatman paper in the following sequence:

3min in 10% SDS, to lyse colonies

7min Denaturation buffer (1.5M NaCl, 0.5M NaCl)

3min Neutralising buffer (1M Tris/HCL pH7.4, 1.5M NaCl)

3min 2xSSC (20xSSC= 3MNaCl, 0.3M NaCitrate)

Filters were air dryed and exposed to UV for 10min to fix the DNA. Cell debris was removed by wiping with 2xSSC/0.1%SDS and the filters were then incubated at 65^{0}C for 1hr in 20ml prehybridisation solution (6xSSC, 0.1%SDS 5xDenhardt's solution's (50 x stock 5g Ficoll, 5g polyvinylpyrolidone, 5g BSA in 500ml dd H_20 filter steralised, aliquoted and stored at -20^{0}C).

1μg of the mutated oligo was labelled with ^{33}P by T4 kinase in a 20μl reaction volume with, 2μl x10 reaction buffer, 4μl ^{32}P γATP (10μCi/μl) and 1μl of enzyme for 1hr at 37^{0}C. The labelled probe was then added to 20ml of hybridisation solution (6xSSC, 5 x 5xDenhardt's solution's) and incubated with the filter for several hours at 10^{0}C below the Tm of the probe (Tm-10^{0}C). Filters were finally washed in 6XSSC (3x 30min, 150ml washes) before exposing to film.

2.B.2 Cell Culture

Cell cultures were grown in a Hereus Cell incubator at 37°C in a humid environment and 5% CO_2. Cultures were grown in Dulbecco's Modified Eagle Medium with 0.11g/l sodium pyruvate with pyridoxine (DMEM) containing 10% foetal calf serum (FCS) and 1% antibiotic/ antimycotic solution (penicillin, streptomycin and amphotericin) in 90mm tissue culture dishes (Nunc). N1E-115 neuroblastoma cell cultures were passaged every 2-3 days by aspirating the media and then physically detaching the cells by repeated pippetting of 10ml fresh media over the dish. The cell suspension was pelleted by centrifugation at 1000g (Beckman GP) for 5 min. The cell pellet was resuspended in fresh media before seeding the cells in fresh dishes. With the use of more adhesive cell lines (Cos-7, Swiss –3T3 fibroblasts and α2.10 chimaerin cell lines), trypsinisation was required to detach cells.

2.B.2.1 Cell stocks

Cell stocks were maintained in liquid nitrogen. Stocks were brought up by thawing the cells rapidly in a 37°C water bath and resuspending in 10ml 10% FCS containing media. After pelleting in a Beckman GP centrifuge (1000g, 5 min) the cells were resuspended in fresh media and seeded out at relevant densities. Cell stocks were constantly replenished:
The plate of cells to be stocked was harvested and pelleted, before resuspending in the freeze stock mixture (90%FCS /10% DMSO). This suspension was then placed in a polystyrene box and placed at –20°C for several hours before placing at –70°C for 16-24-hrs and finally in liquid nitrogen.

2.B.3 Morphological Analysis

2.B.3.1 Coverslip preparation

For morphological analysis, cells were seeded on coverslips:

Glass coverslips (22mm x 22mm) were cleaned with a 30 min wash in 60% Ethanol/40% HCl solution. After washing in copious amount of water, coverslips were dried and heat sterilised (4 hours at 121°C). Glass coverslips could be used 'bare' or coated with poly(L) Lysine. A solution of poly-L-lysine (5µg/ml) was used to coat the coverslips for 30min at room temperature before excess protein was washed away with ddH_2O. Prepared coverslips were placed in 30mm dishes and cells were seeded onto them ($1.3x10^5$), in 2ml of 10% FCS containing media.

2.B.3.2 Transfections on coverslips

To examine the morphological effect of over expression of proteins, cells were transiently transfected with relevant cDNAs. Cells were serum starved for 1hr in 1ml DMEM media, while the transfection mix was prepared. The relevant cDNAs (1ug), lipofectamine 2000 (6ul) and 200µl DMEM were mixed and incubated at room temperature for 45min. This mix was then added to the cells and after 5hrs the media was replaced with fresh FCS and antibiotic containing media. Proteins were allowed to express for 16hrs.

2.B.3.3 Immunostaining

Cells, on coverslips were washed briefly in PBS before fixing in 4% paraformaldehyde/PBS solution for 10min. A 10min wash in PBS was carried out before quenching residual PFA in 100mM Glycine/PBS for 10min. Cells were then permeabilised with 0.2% Triton-x-

100/PBS for 10min and blocked in 3% BSA/PBS for a further 10min. Incubation with the relevant antibodies in 1% BSA/PBS solution was carried out in a humid container for 1-2hrs at 37^{0}C. Excess antibody was washed away with 3 x 5min PBS washes before incubation with the relevant FITC/TRITC/Cy5 conjugated secondary antibodies/phalloidin for 1hr at 37^{0}C. Again 3 x 5min washes in PBS were carried out and finally the coverslips were air dryed before mounting on glass slides with a drop of immuno fluorescent mountant. Slides were left to air dry at room temperature and then stored at 4 ^{0}C in the dark.

2.B.3.4 Fixed cell imaging and characterisation

Slides were examined using a Zeiss axioplan microscope. 300 transfected cells per experiment were counted for various phenotypes and three separate experiments performed for each experiment. The student t test was performed to determine statistical significance. Cell images were taken using a Zeiss LSM 4.10 Confocal microscope.

2.B.3.5 Live cell imaging

For live cell fluorescent imaging, cells were transfected on glass bottom dishes (World Precision Instruments) before imaging in a heated humid chamber with a Zeiss axiovert 135 microscope and Cool SNAP camera (Photometrics).

2.B.4 Protein Work

2.B.4.1 Recombinant protein production

Recombinant proteins were expressed and isolated in BL-21cells which are deficient in a number of proteases, allowing greater yields of protein production. pGEXcDNAs were used, containing an inducible promoter, allowing the production of GST fused proteins. BL-21s were transformed and grown up as per XL-1 Blue cells. 500ml cultures were inoculated from overnight cultures (1/20) and grown at 32^0C with vigorous shaking. When the $O.D_{595}$ of the culture reached 0.5-1, protein expression was induced with the addition of IPTG (0.2mM). Expression was continued for 2hrs at 32^0C before the cells were harvested by centrifugation, washed in PBS, repelleted and frozen at -70^0C.

Temperatures were kept at a minimum (on ice) throughout the isolation procedure to reduce the action of proteases. Cells were thawed and resuspended in 20ml of lysis buffer (Tris/HCL pH8, 1%Triton-X-100. 1mM PMSF + Complete protease inhibitors). Lysate was sonicated (level 6, 20s pulses) until clear (~2min) and incubated on ice for 20min. Cell lysate was cleared by centrifugation at 20Kg for 20min and to the supernatant 100µl/100ml culture of prewased 4B beads (3x 40ml PBS) were added and incubated at 4^0C for ~2hrs on a spiral mixer. The beads were washed 3 x 30min in 40ml lysis buffer, pelleting each time by centrifugation (5min, 3000g).

The following steps in the isolation procedure depends on the desired recombinant protein:

2.B.4.1.1 GST fused protein

Beads were washed in 50mM Tris/HCL pH 8 before incubating at room temperature for 15min with 15ml elution buffer (10mM Glutatione/50mM Tris/HCL pH8). Beads were removed by passing the slurry through a column and the elutant was dialysed in PBS (3x 2L) at 4^0C.

2.B.4.1.2 Non tagged protein:

For a pure protein, cleavage from the GST moiety using thrombin was carried out. Beads were washed in Thrombin Cleavage buffer (50mM Tris/HCl pH7.5, 150mM NaCl, 3mM $CaCl_2$) before incubating with 5 units of thrombin per expected mg protein o/n at 4^0C in 10ml Thrombin Cleavage buffer. The slurry was passed through a column where the filtrate was incubated with 0.5ml Benzamidine for 15min at 4^0C to remove the thrombin. After passing through another column, the isolated protein could be concentrated using Amicon's Centriprep centrifugal filter devices.

2.B.4.2 Mammalian cell 90mm plate transfection

For *in vivo* analysis and the isolation of mammalian expressed proteins, cells were transiently transfected with the relevant cDNAs. Sub confluent (~70%) mammalian cells in 90mm dishes were serum starved for 1hr in 5ml DMEM. The transfection mix was prepared during serum withdrawal by mixing the relevant cDNAs (5μg) with 20μl lipofectamine in 1.3ml DMEM. This transfection prep was incubated at room temperature for 45min before adding to the cells. 5hr after addition, the media was replaced with fresh 10% FCS, 1% antibiotic containing media and proteins were allowed to express for 16-48 hrs.

2.B.4.3 Cell treatment

Numerous *in vivo* treatments were carried out before protein isolation and analysis

2.B.4.3.1 Pervanadate (peroxides of vanadate)

A powerful inhibitor of tyrosine phosphatases, pervanadate is a useful tool to investigate phosphotyrosine signalling (Kadota., et al 1987). Pervandate was prepared by mixing 60µl sodium orthovanadate (Na_3VO_4 200mM), 539µl PBS and 1µl 30% w/w H_20_2 and incubating at room temperature for 5 min. Excess H_20_2 was removed by the addition of 200ug/ml catalase (0.6µl 20mg/ml stock) and incubating at room temperature for a further 5 min. Peroxide treated pervanadate was used immediately treating as x200 stock (100µM final) in DMEM for 20 min at 37^0C.

2.B.4.3.2 Phorbol 12-myristate 13-acetate (PMA)

PMA (Sigma) is a mimic of DAG and was used to study the phorbol ester receptor α2 chimaerin. PMA was made up in DMSO (1M) and cells were treated at the relevant concentration for 1hr at 37^0C in relevant media.

2.B.4.4 Cell lysis

Mammalian cells, in 90mm culture plates and expressing the relevant plasmids or treated with the relevant conditions, were washed in PBS and then lysed on the dish with 500µl lysis buffer (Tris-HCL pH7.5, 0.3M NaCl, 1% Triton-x-100, 1mm $NaVO_4$, 5mm DTT, 1mM PMSF) on ice for 5 min. Cells lysate was collected by scraping and this was solubilised

with sonication at level 6 for 1 min (20sec pulses/5sec rest) and incubating on ice for 30min. The cell lysate was cleared by centrifugation at 20Kg for 20min.

2.B.4.5 Immunoprecipitation/GST pulldown

GST pulldowns, using sepharose 4B beads, and agarose conjucated antibody immunoprecipitations (Sigma FLAG-M2, Cdk5-AC) were carried out as follows.

450ul of the cleared lysate was added to 20µl of pre-washed agarose conjugated/ 4B beads (washed 2x 1ml Lysis buffer) while the remaining lysate was kept for analysis of protein expression. The beads were incubated at 4^0C for 2-3hrs on a rotor, before washing with repeated cycles of pelleting/resuspending (centrifugation at 1kg for 1min/ resuspending in 1ml of lysis buffer for 2-3min). After washes, the captured proteins were eluted by heating to 95^0C for 5 min in 30ul 2x sample buffer.

With non agarose conjugated antibodies, immunoprecipitations were carried out in a similar manner: 5µg of antibody was added to 450ul of the cleared lysate. After 1hr incubation at 4^0C on a rotor, 30µl of rehydrated/washed Protein A-beads (0.05g in 1ml H_20, wash 3x 1ml H_20) was added to capture the antibody. Incubation at 4^0C was carried out for a further 2hrs before washing and eluting as before.

2.B.4.6 Rac-GTP assay

To determine the amount of active Rac (GTP bound) in a cell, PAK's CRIB (Cdc42 and Rac interactive binding) domain was utilised, which only associates with GTP bound p21s. Recombinant CRIB was grown up and isolated as described but left attached to 4B beads.

Cells were washed with ice cold PBS + 1mM $MgCl_2$ + 0.5mM $CaCl_2$ before quickly harvesting on ice with 0.5ml Lysis buffer (Tris-HCL pH7.5, 0.3M NaCl, 1.5% Triton-x-100, 1mm $NaVO_4$, 5mm DTT, 1mM PMSF). Lysate was rapidly cleared with 2min 20,000xg spin down before 450ml of cleared lysate was added to relevant amount of bound GST-PAK CRIB. The beads were incubated at 4^0C for 1-2hrs on a rotor, before washing with repeated cycles of pelleting/resuspending (centrifugation at 1000xg for 1min/ resuspending in 1ml of lysis buffer for 2-3min). After washes, the captured proteins were eluted by heating to 95^0C for 5 min in 30ul 2xlamelli sample buffer.

2.B.4.7 Cell fractionation

For analysis of α2 chimaerin's subcellular distribution, α2.10 Cells were plated out on 50mm dishes (4 $x10^5$) in 10%FCS containing media and cultured overnight. Cells were washed in PBS before lysing and harvesting in 200μl Hypotonic buffer (Tris/HCL pH7.5 2mM PMSF) off the plate. The cell lysate was completely suspended by sonication at level 6 for 1.5 min (20sec pulses/5sec rest) and then cleared by centrifugation at 49Kg for 1hr at 4^0C(Beckman TL-100). The supernatant (Cytosolic fraction) was collected and stored and the pellet was washed twice with 200μl hypotonic buffer. The pellet was then resuspended in 200μl 1% Triton buffer (1% Triton-x-100, Tris/HCL pH7.5, 2mM PMSF) and again completely resuspended by sonication at level 6 for 3 min (20sec pulses/5sec rest). After

incubation on ice for 15min the cell lysate was centrifuged at 18,000g, for 20min, at 4^0C in a benchtop centrifuge. The supernatant (Triton soluble fraction) was again removed and stored and the pellet was washed twice with 200µl 1%Triton buffer. This final pellet was resuspended in 50µl 2x Lammelli sample buffer (Triton insoluble fraction) by extensive sonication (level 9) and freeze thaw cycles.

2.B.4.8 *In vitro* kinase assays

In-vitro kinase assays were carried out on recombinant protein substrates or immunprecipitated proteins. 2-5µg substrate protein was suspended in the relevant kinase buffer in a 30µl reaction volume. The required amount of kinase plus ATP (100µM final concentration) and 0.37MBq of ^{32}P γATP (111TBq/mMole) was added and the reaction incubated at 30^0C for 30min, before quenching with 15µl of Laemmli Sample Buffer and heating to 95^0C for 5min.

2.B.5 Protein Analysis

2.B.5.1 Protein quantification

The amount of protein in a sample was evaluated using Biorad protein reagent. 200µl Biorad reagent, a relevant amount of the sample (1-5µl) and ddH_20 to a final volume of 1ml were mixed together and incubated at room temperature for 15min. The O.D at 595nm wavelength was recorded and by comparison to BSA standards the amount of protein evaluated.

2.B.5.2 SDS-polyacrylamide gel electrophoresis

SDS polyacrylamide gel electrophoresis was used to separate proteins based on their molecular weight. The acrylamide concentration in the resolving gel is determined by the molecular weight of the proteins to be resolved. Typically 10% acrylamide resolving gels were used for the separation of proteins 16kDa to 68kDa KD:

3.15ml 30% acrylamide/bis-acrylamide, 1.25ml 1.5M Tris/HCl pH8.8, 1.25ml 80% Glycerol, 4ml H_20, 100µl 10% (w/v) SDS, 50µl 10% APS, 5µl TEMED.

7.5mls of the gel solution was pipetted into a mini gel apparatus (Biorad) and overlayed with ddH_2O, to prevent any oxidation. The gels were left to polymerise at room temperature for 45 minutes after which the ddH_2O was poured off and a stacking gel mixture was prepared:

1.67ml 30% acrylamide/bis-acrylamide,1.25ml 0.5M Tris/HCl pH6.8, 7ml ddH_2O,100µl 10% (w/v) SDS, 50µl 10% APS, 12µl TEMED

The stacking gel was pipetted on top of the polymerised resolving gel, into which a teflon comb was inserted. The stacking gel was left to polymerise at room temperature for 30 minutes before removing the comb.

Samples were mixed with 5 x SDS Laemmli gel loading buffer (2% (v/v) SDS, 10% (v/v) glycerol, 62.5mM Tris/HCl pH 7-8, 5% (v/v) β–mercaptoethanol, bromophenol blue) and heat denatured at 95°C for 5 minutes before loading onto the denaturing SDS polyacrylamide gels. To determine the molecular weight of proteins separated on the acrylamide gels, protein markers of known size were also run. Proteins were separated using a Biorad vertical gel discontinuous buffer system. Gels were run at 170V for 1 hour in 1 x electrode buffer (10 x electrode buffer: 0.25mM Tris, 0.5M Glycine, 1% SDS).

Proteins could be either transferred onto PVDF membranes (western transfer) or visualised by Commasie blue staining:

2.B.5.3 Commasie blue staining

To stain for proteins in gels the gels were soaked for several hours in Commasie blue solution (40% Methanol, 10% glacial acetic acid, 0.005% Commasie brilliant blue). Excess Commasie blue was removed (destained) by soaking in 40% Methanol, 10% glacial acetic acid solution until background levels were negligible.

2.B.5.4 Western transfer (semi-dry blotting)

Proteins separated by electrophoresis were transferred onto PVDF membranes for antibody probing. The SDS-gels were equilibrated in 1 x transfer buffer (100ml of 10 x stock (25mM Tris, 192mM glycine) 20% (v/v) methanol made up to 1 litre with ddH_2O), along with a piece of PVDF membrane (PVDF was pre-soaked in methanol and washed in ddH_2O before equilibration) and two pieces of extra thick and 3mm Biorad filter paper. A sandwich was prepared on a Biorad Transfer-Blot SD semi-dry transfer machine, ensuring no air bubbles were trapped: Bottom-Top: Thick Biorad filter paper, Whatman filter paper, PVDF membrane, SDS-acrylamide gel, Whatman filter paper and thick Biorad filter paper. Proteins were transferred overnight at a constant voltage of 8 volts.

2.B.5.5 Immunoblotting

After western transfer, the PVDF was allowed to air dry at room temperature to ensure maximal protein immobilisation. The membranes were then stained/destained with Commmasie blue to visualise the immobilised proteins (also reduces background of immunoblotting). After ddH_20 washes the membranes were incubated in blocking solution (5% marvel milk, 0.1% tween-20/PBS) for 1hr at room temperature. Membranes were incubated with the required antibodies at the relevant concentration in 1% marvel, 0.1% tween-20/PBS for 1-2hrs at room temperature. Excess antibody was washed away with 5 x 5min, 10ml 0.1% tween-20/PBS washes. The primary antibody could then be detected using a secondary horseradish peroxidase (HRP) conjugated antibody (Dako) raised against the Fc portion of the IgGs from the species of the primary antibody. A 1/2000 dilution in 1% marvel, 0.1% tween-20/PBS was used for 1hr at room temperature. Again excess antibody was washed away with 0.1% tween-20/PBS washes. Bound secondary antibody was detected by enhanced chemi imunofluorescence (ECL Amersham). Equal volumes of reagents 1 and 2 were mixed and washed over the membranes for 1min. Excess fluid was removed with tissue paper and the membranes were exposed to ECL film for developing.

Chapter Three

Results I

3 α2 Chimaerin: GAP Regulation and Neuronal Morphology

The Rho GTPases have been implicated in numerous cell responses and their cycling activity is crucial for function. The C-terminus of α chimaerins contain a GAP domain, which is selective towards Rac 1 (Diekeman et al., 1991, Manser et al., 1992). α Chimaerins thus down regulate Rac 1 activity but could also promote Rac signalling (Kozma., et al 1996) possibly via coordinating with a GEF to increase cycling or via an effector function.

Two α chimaerin isoforms exist, α1 (Hall et al., 1990) and alternatively spliced α2 (Hall et al., 1993), containing a SH2 domain at its N-terminus. A cysteine rich domain (C1) and GAP domain are also common to both isoforms (see 1.G.2.1). The C1 domain is homologous to the C1 region of PKCs and is required for phospholipid-dependent phorbol ester binding (Ahmed et al., 1990). Phospholipid association has been shown to regulate α chimaerin GAP activity *in vitro* (Ahmed et al., 1993).

As well as differences in developmental expression patterns, α chimaerin isoforms differ in their subcellular distribution, with α2 chimaerin adopting a more cytosolic distribution compared with the predominant membranous/cytoskeletal association of α1chimaerin (Hall et al., 2001). This different cellular distribution of α2 chimaerin has been presumed to be due to the SH2 domain, suggested by a "translocating" mutation within the SH2 domain (N94H), which results in an α1 chimaerin–like distribution and morphology in N1E-115 neuroblastoma cells.

It can thus be hypothesised that signalling events altering α2 chimaerin location/lipid association would in turn regulate GAP activity and the effect of this on neuronal cell morphology is of interest.

3A. α2 Chimaerin Location

3.A.1 Mutations in the SH2 and GAP domains of α2 chimaerin alter subcellular distribution

Modelling α2 chimaerin SH2 domain on Grb2 SH2 domain structure indicated the N94H mutation could potentially locally alter surface charges in the region adjacent to the phosphotyrosine binding pocket, without directly effecting phosphotyrosine binding. It is not clear whether this mutation affects interactions with target proteins or intramolecular interactions. It was of interest to determine if mutations in other regions of α2 chimaerin might result in altered distribution. The sub-cellular distribution of α2 chimaerin mutants was examined in Cos-7 cells transiently transfected with GFP fused α2 chimaerin cDNA constructs (Fig. 3.1).

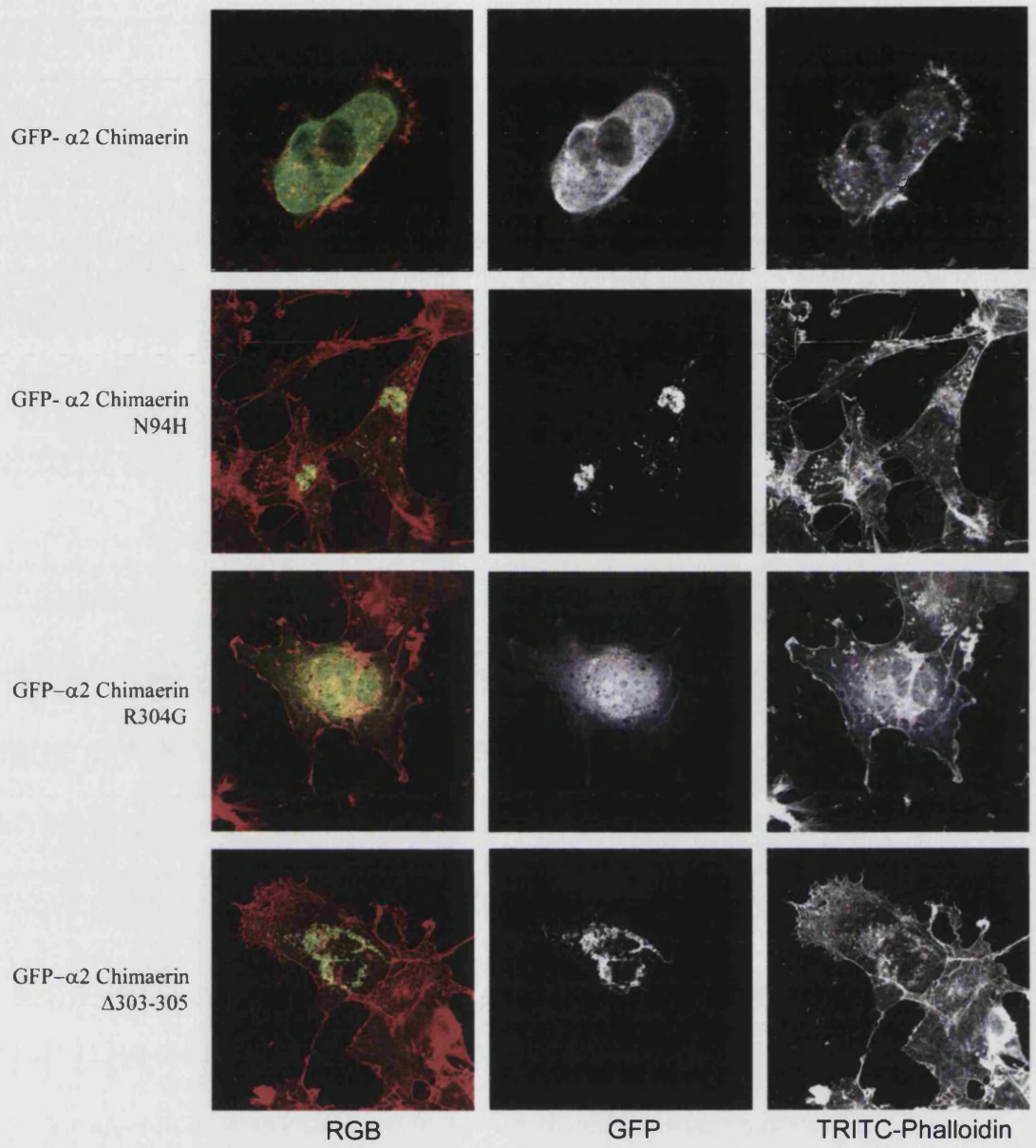


Fig. 3.1 α2 chimaerin mutant expression in Cos-7 cells.

Cos-7 cells were transiently transfected on glass coverslips, with indicated GFP tagged α2 chimaerin cDNA constructs (green) and cultured in 10% FCS containing media for 16 hrs before fixing and staining with TRITC-phalloidin (red).

In Cos-7 cells, the transient expression of the Rac GAP, α2 chimaerin, surprisingly had little effect on cell morphology, as Rac-dependent, lamellipodial structures were still present. α2 chimaerin was uniformly distributed throughout the cytosol and extended to peripheral structures (Fig. 3.1 Top row). However deletion of three residues in the GAP domain of α2 chimaerin (Δ303-305) (Fig. 3.1 4th row), resulted in a more particulate

distribution with the mutated protein concentrating around the nucleus, similar to that seen with α2 chimaerin N94H (Fig. 3.1 2nd row). This distribution indicates insolubility of the mutant proteins through membrane association or possibly through protein aggregation. The deletion of Δ303-305 in α1 chimaerin, eliminates GAP activity but maintains Rac binding (Ahmed et al., 1994). Arginine 304 constitutes a highly conserved residue in the GAP domain, whose mutation has been shown to abolish GAP activity (Nassar et al., 1998). α2 chimaerin R304G maintained normal α2 chimaerin distribution implying this difference between Δ303-305 and R304G is independent of GAP activity. Similar results were obtained when these mutants were expressed in N1E-115 neuroblastoma cells (see Fig 3.8). The association with internal membranes and not the plasma membrane, by the translocating mutants, is consistent with β2-chimaerin distribution in Cos-1 cells, where Golgi localisation was observed (Caloca et al., 2001).

The altered distribution of the mutants could result from conformational alterations in α2 chimaerin. α2 chimaerin may adopt a closed conformational structure, in which C1 domain mediated membrane insertion is inhibited, maintaining α2 chimaerin in the cytosol. It is tempting to speculate the closed conformational could be a result of an intramolecular interaction between SH2 and GAP domains with a key Tyr residue, Tyr^{303}. This however remains to be determined by mutational analysis.

3.A.2 PMA translocates α2 chimaerin

The C1 domain of chimaerin is responsible for DAG/phorbol ester binding (Ahmed et al., 1990). Phorbol ester treatment has been shown to translocate PKC to membranes, with the accepted view that ligand binding to the C1 domain caps a hydrophilic site at the top of the structure; providing a contiguous hydrophobic surface, promoting

insertion into the lipid bilayer (Zhang et al., 1995). It has been demonstrated that α1, β1 and β2 chimaerin are all translocated to membranous/cytoskeletal compartments upon phorbol ester treatment in Cos-1 cells (Caloca et al., 1997). In this study the functional consequence of phorbol ester treatment on α2 chimaerin was investigated in relevant cell types.

α chimaerins are neuronally expressed proteins (Hall et al., 1990, Hall et al., 1993), thus to evaluate *in vivo* functions of α2 chimaerin, a permanent N1E-115 neuroblastoma cell line over-expressing α2 chimaerin was utilised, α2.10 line (Hall et al., 2001). These cells adopt a more flattened phenotype with prominent lamellipodia, compared with parental N1E-115 cells. A proportion of these cells also form neurites in 10% serum containing media and they are more adhesive, requiring trypsinisation during culturing. α2.10 cells were treated with the phorbol ester, PMA, and fractionated (A) or fixed and stained with α2 chimaerin antibody (B).

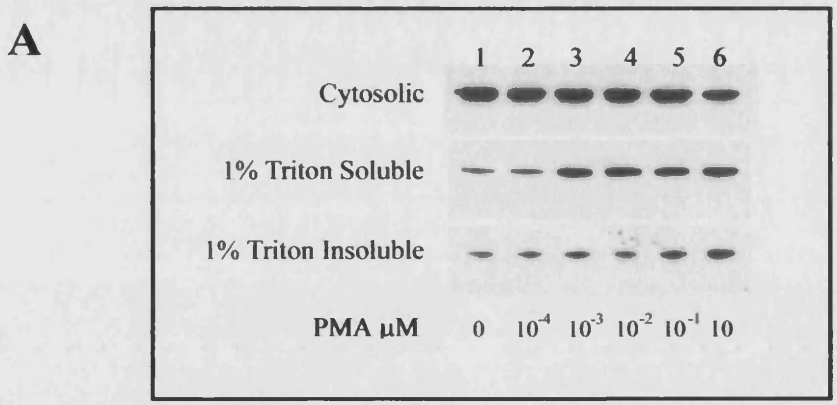


Fig. 3.2 PMA translocates α2 chimaerin in neuroblastoma cells.

α2.10 neuroblastoma cells were treated with PMA in 10% FCS containing media for 1 hr. Cells were fractionated and 25 μg of cytosolic, 1% Triton soluble proteins, and 10 μl of 1% Triton insoluble fraction were analysed by SDS gel electrophoresis and immunoblotting with α2 chimaerin antibody (A).

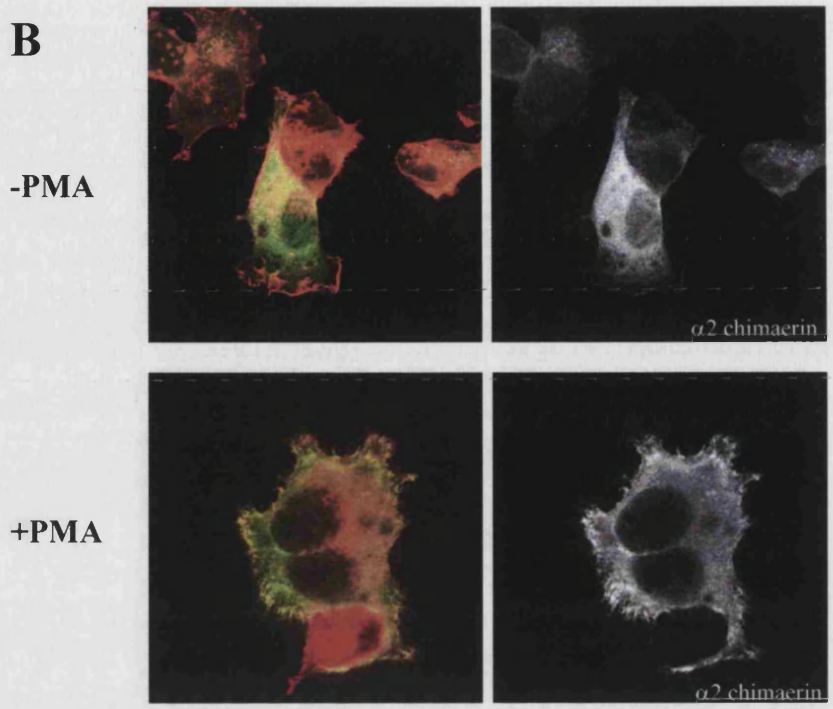


Fig. 3.2B PMA translocates α2 chimaerin in neuroblastoma cells.
α2.10 neuroblastoma cells were treated with 10 μM PMA, or vector, in 10% FCS containing media for 1 hr. Cells were fixed and stained with TRITC phalloidin (red) and α2 chimaerin antibody (green)

From cell fractionation and immunoblotting, a clear translocation of α2 chimaerin from the cytosol to 1% Triton soluble and insoluble fractions was found to occur, upon PMA treatment, with concentrations as low as 10 nM being effective (Fig. 3.2A lane 3). Cell staining of α2.10 neuroblastoma cells showed α2 chimaerin adopts a roughly uniform distribution throughout the cytosol, extending to peripheral structures such as lamellipodia, where α2 chimaerin stains the actin rich leading edge (Fig. 3.2B). PMA induced translocation results in a particulate accumulation concentrating near the cell periphery. This implies possible translocations to membranes and cytoskeletal components as well as lipid rafts upon PMA treatment. A dramatic change in neuroblastoma cell morphology was also observed upon PMA treatment. The flattened, spread cells (indicative of Rac activity) became contracted with loss of lamellipodia and the appearance of microspikes, consistent with down regulation of Rac activity.

3.B α2 Chimaerin GAP Activity and Neuroblastoma Cell Morphology

3.B.1 PMA causes retraction of α2.10 neuroblastoma cells accompanied by a decrease in Rac-GTP

It appeared after fixing and staining that α2.10 neuroblastoma cells had responded to PMA treatment with altered morphology; this was therefore monitored with live cell imaging.

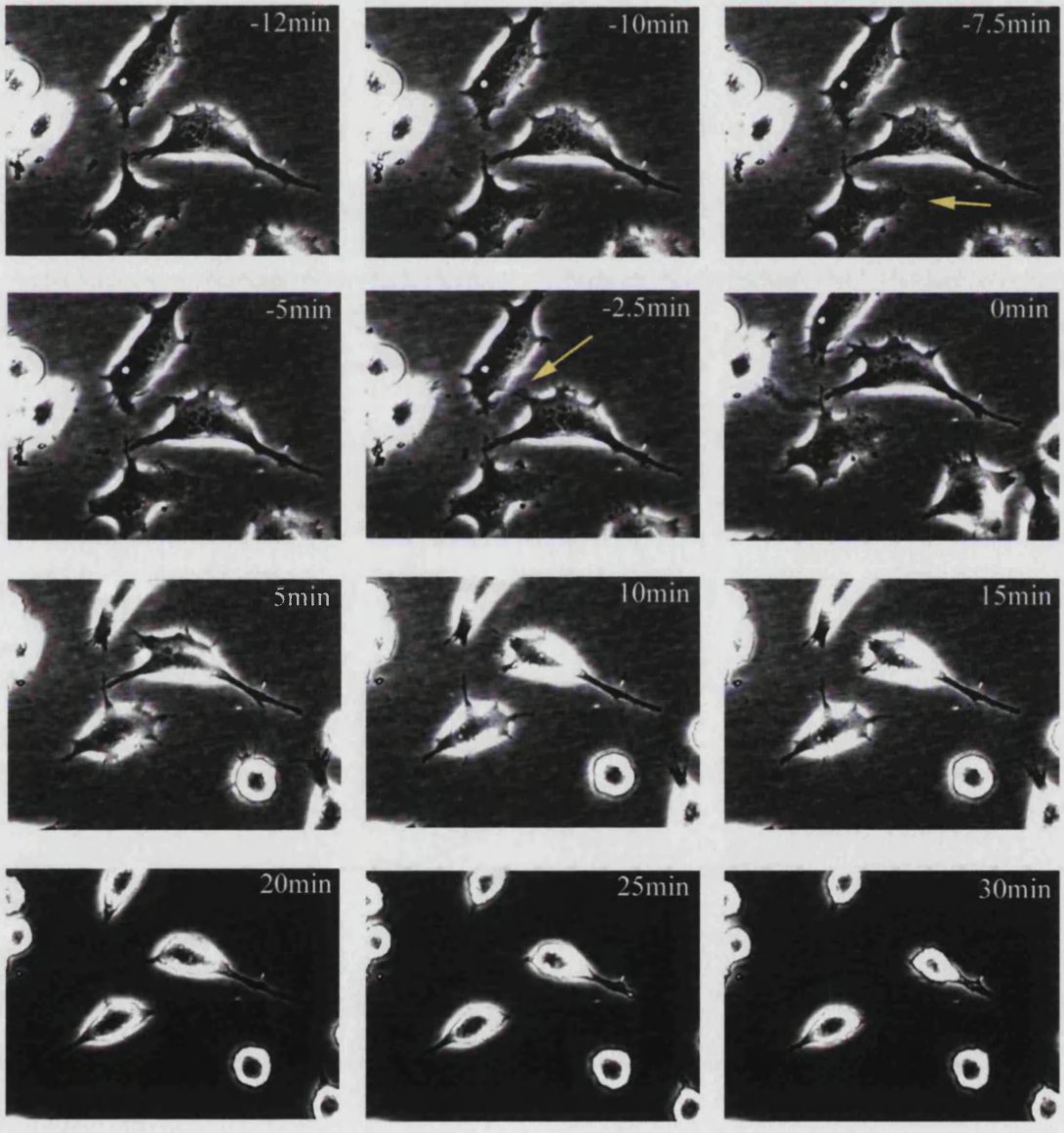


Fig. 3.3 PMA induced retraction of α2.10 neuroblastoma cells.
α2.10 neuroblastoma cells, seeded on poly-L-lysine in 10% FCS containing media, were treated with 10 μM PMA and monitored by phase contrast microscopy with images recorded over the indicated time intervals.

Neuroblastoma cells permanently over-expressing α2 chimaerin (α2.10) generate neurites in the presence of 10% FCS (Hall et al., 2001). Live cell imaging phase contrast analysis showed these cells were morphologically very dynamic, exhibiting fluctuating lamellipodia (arrows -7.5 and -2.5 min prior to PMA treatment), indicative of dynamic Rac activity (Fig. 3.3). This is consistent with results obtained by microinjection of a recombinant α1 chimaerin protein into neuroblastoma cells where dynamic Rac/Cdc42 effector functions were apparent (Kozma et al., 1996). Upon PMA treatment of α2.10 neuroblastoma cells, rapid retraction/collapse was observed. The microspikes observed in fixed neuroblastoma cell staining (Fig. 3.2B) were therefore likely to be retraction fibres rather than filopodia formation.

In Swiss 3T3 fibroblast cells, PMA treatment has been shown to induce prominent lamellipodia, indicating activation of Rac signalling (Ridley et al., 1992). The lack of this response to PMA in the α2.10 neuroblastoma cell line was thus intriguing. The Rac activity in these cells in response to PMA was determined using a GST recombinant PAK CRIB domain fusion protein, immobilised on Sepharose beads to pull out Rac-GTP.

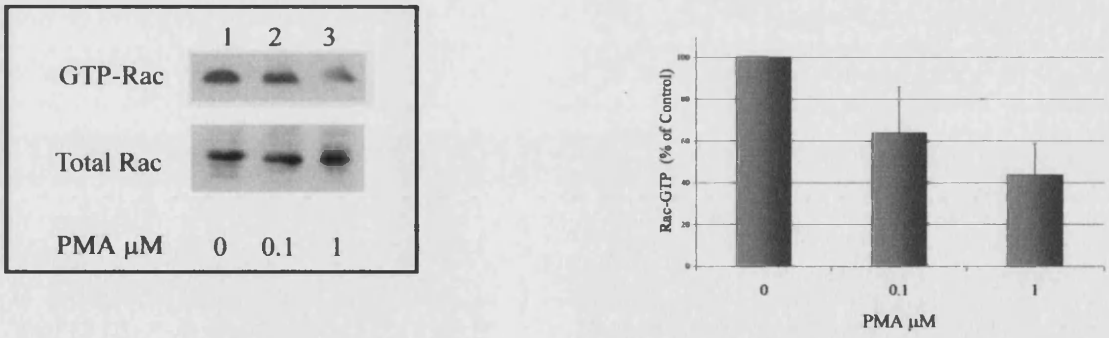


Fig. 3.4 α2.10 neuroblastoma cell Rac signalling, in response to PMA.

α2.10 neuroblastoma cells in 10% FCS containing media were treated with indicated concentrations of PMA for 1hr before harvesting and extracting Rac-GTP with GST-PAK CRIB domain pull downs. Western blots of total and pulled out (GTP) Rac were densitometry analysed and Rac-GTP levels as a percentage of the control (no PMA) were evaluated (error bars indicate SDs for three separate experiments).

There was a decrease in Rac-GTP levels in these neuroblastoma cells permanently expressing α2 chimaerin with increasing concentration of PMA treatment (Fig. 3.4). This decrease in Rac-GTP is consistent with activation of α2 chimaerin GAP activity.

3.B.2 PMA causes retraction of N1E-115 neuroblastoma cells which can be inhibited by the expression of GAP inactive α2 chimaerin

The lack of N1E-115 neuroblastoma cell ruffling in response to PMA has previously been reported (Kozma et al., 1997). N1E-115 neuroblastoma cells endogenously express α2 chimaerin and thus it was of interest to determine if there was a similar morphological response in parental cells to PMA treatment, which could be attributed to α2 chimaerin GAP activity. GAP inactive α2 chimaerin mutants, which maintain Rac binding, can act as dominant negative constructs *in vitro* (Ahmed et al., 1994). N1E-115 neuroblastoma cells deprived of serum undergo Rac dependent morphological differentiation and neuritogenesis (Kozma et al., 1997) providing an *in vivo* system to examine α2 chimaerin Rac GAP activity.

N1E-115 neuroblastoma cells were plated onto poly-L-lysine and transiently transfected with GFP-α2 chimaerin constructs, before inducing differentiation by culturing in low serum containing media. Cells were then treated with PMA, for up to 30min, fixed, stained and the number of transfected cells bearing neurites counted.

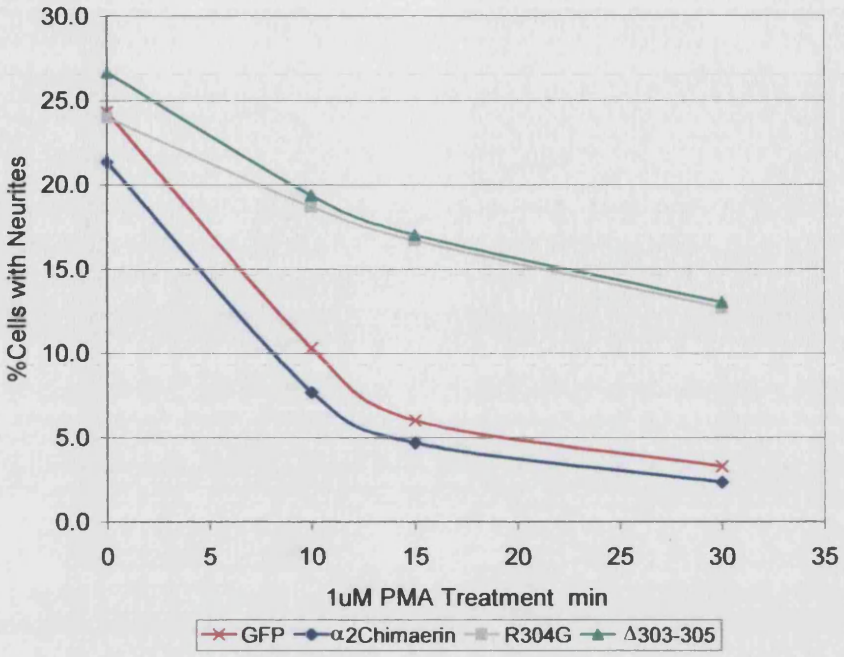


Fig. 3.5 Inhibition of PMA induced retraction of neuroblastoma cells by expression of α2 chimaerin GAP inactive mutants. N1E-115 neuroblastoma cells were transiently transfected with GFP, GFP-α2 chimaerin, GFP-α2 chimaerin R304G or GFP-α2 chimaerin Δ303-305 constructs and grown in 1% serum media for 16 hrs. Cells were treated with 1 μM PMA for indicated times, before fixing and staining with TRITC-phalloidin. Cells with processes > 2 cell body diameters were counted as neurite bearing cells.

Culturing N1E-115 neuroblastoma cells in media containing low serum (1% FCS) induces Rac/Cdc42 dependent neurite formation (Kozma et al., 1997) with ~25% cells bearing neurites. Subsequent PMA treatment caused obvious neurite retraction of both GFP and GFP-α2 chimaerin over-expressing neuroblastoma cells within 15 min (Fig. 3.5). This PMA induced retraction was significantly reduced in cells expressing the GAP inactive mutants ,GFP-α2 chimaerin R304G and Δ303-305.

These GAP inactive mutants are capable of Rac association and thus potentially act as dominant negative constructs, inhibiting endogenous α2 chimaerin GAP activity through competition of Rac and DAG associations (Ahmed et al., 1994). Lack of complete effectiveness implicates incomplete inhibition by the transfected proteins or possibly alternative signalling pathways, potentially PKC mediated. To examine a

possible role for PKC in this PMA induced response, cells were pre-treated with the PKC (α,β_{II},β_I,γ,δ and ε) kinase inhibitor GF109203X.

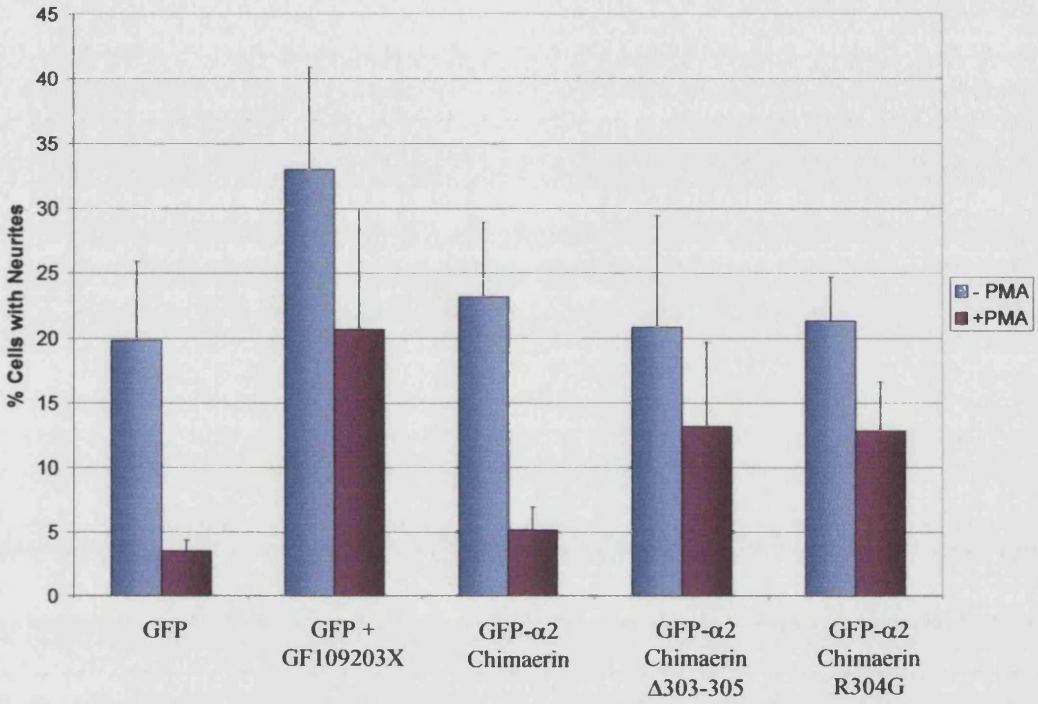


Fig. 3.6 PKC involvement in PMA induced neuroblastoma cell retraction.
N1E-115 neuroblastoma cells were transiently transfected with indicated GFP tagged constructs and grown in 1% serum media for 16hrs. Cells were pre-treated +/- PKC inhibitor (5 μM GF109203X) for 30 min, before 15min incubation in +/- 1μM PMA. Cells were fixed and stained with TRITC-phalloidin and cells possessing processes > 2 cell body diameters were counted as neurite bearing cells (error bars indicate SDs for three separate experiments)

None of the GFP fused α2 chimaerin constructs significantly altered the basal level of neurite formation upon serum withdrawal, with no observed increase in neurite length. Thus none of these constructs inhibited Rac dependent outgrowth, either through possible sequestration or down regulation of Rac. This suggests an inactive state of the expressed wild type α2 chimaerin. Treatment with the PKC kinase inhibitor, GF109203X, caused dramatic filopodia formation with significantly increased neuritogenesis compared with control cells ($p<0.05$), within the short period of

treatment (45min). Thus it appears PKC kinase activity serves to inhibit filopodia and neurite formation in N1E-115 neuroblastoma cells.

Over-expression of α2 chimaerin did not significantly enhance the retraction of neurites upon PMA treatment but expression of both GAP inactive α2 chimaerin mutants, Δ303-305 and R304G, did reduce collapse (37% and 40 % collapse compared to control GFP collapse of 82%). No significant retraction was observed in cells expressing GFP α2 chimaerin Δ303-305 upon PMA treatment ($p>0.1$) and although cells expressing α2 chimaerin R304G did significantly retract in response to PMA this response was small compared to GFP and GFPα2 chimaerin expressing cells. PMA in the presence of GF109203X also did not cause significant collapse ($p>0.05$, 37% collapse). However GF109203X treatment induced significant neuritogenesis compared with GFP expressing cells alone ($p<0.05$). Thus inhibition of both α2 chimaerin and PKC activities separately can reduce PMA induced retraction of neurites to some degree, implicating both these proteins in this PMA induced response. Of interest is the similar degree of inhibition produced by PKC inhibitor and GAP inactive constructs, which might implicate both proteins in the same signalling pathway. To investigate this further cells transfected with GAP inactive α2 chimaerin constructs, treated with PMA in the presence of GF109203X would need to be evaluated.

These results obtained by expression of GAP inactive constructs imply α2 chimaerin GAP activity is not required for neurite outgrowth stimulated by serum withdrawal, but is implicated in PMA induced neurite retraction of N1E-115 neuroblastoma cells. PKC also appears involved in this morphological response to PMA and inhibition of PKC kinase activity causes increased neuritogenesis (at least as a short term response). However caution must be taken regarding the specificity of inhibitors. GF109203X is a potent inhibitor of GSK3 (Hers et al., 1999), as well as PKC and

therefore GSK3 and other potential kinase activities can not be excluded from the observed results.

3.B.3 α2 chimaerin GAP activity inhibits neuritogenesis of N1E-115 neuroblastoma cells when membrane targeted

PMA treatment appears to activate α2 chimaerin GAP activity and induce neurite retraction. To further investigate the effect of α2 chimaerin GAP activity on neurite morphology, N1E-115 neuroblastoma cells on poly-L-lysine were transiently transfected with GFP fused α2 chimaerin constructs and cultured in low serum media before fixing and staining cells.

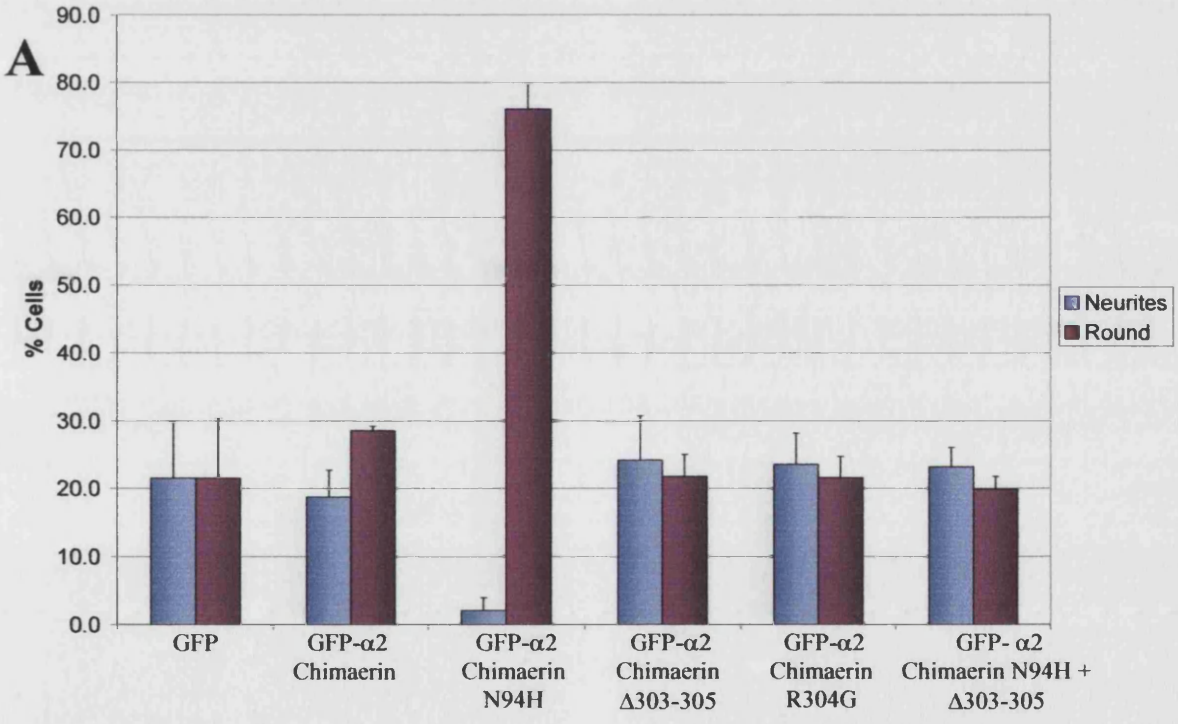


Fig. 3.7 α2 chimaerin GAP activity inhibits neuritogenesis of N1E-115 neuroblastoma cells. N1E-115 neuroblastoma cells on poly-L-lysine were transfected with the relevant GFP constructs and cultured in 1% FCS containing media for 16hrs before fixing and staining with TRITC- phalloidin (B). Transfected cells with processes >2 cell body diameters (neurite bearing) and cells possessing no visible processes (round) were counted (A) (error bars indicate SDs for three separate experiments).

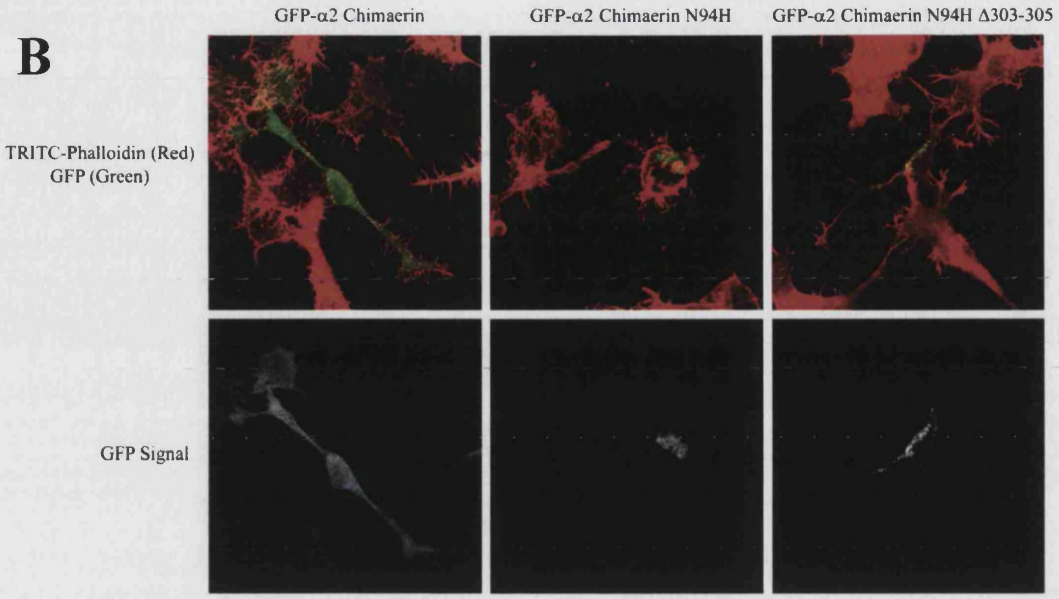


Fig. 3.7B α2 chimaerin GAP activity inhibits neuritogenesis of N1E-115 neuroblastoma cells.

Serum withdrawal of neuroblastoma cells results in neuritogenesis with approximately 20% of GFP expressing control cells bearing neurites under these low serum conditions (Fig. 3.7A). The over-expression of wild type α2 chimaerin and GAP inactive mutants (R304G and Δ303-305) did not significantly affect this process of neurite formation compared with control GFP transfected cells ($p>0.1$), implying α2 chimaerin GAP activity is not required for neurite outgrowth of N1E-115 neuroblastoma cells. However, expression of α2 chimaerin N94H, which adopts a membranous localisation, almost completely inhibits neurite formation compared with control GFP cells ($p<0.01$), causing cells to round up ($p<0.001$). This inhibition of neurite outgrowth is a direct result of GAP activity as the use of the double mutant (α2chimaerin N94H Δ303-305) restored normal neurite formation (Fig. 3.7 A + B).

From the cell staining, α2 chimaerin is found uniformly throughout the cytoplasm extending along neurites and even to filopodia structures (Fig.3.7B). The double mutant α2 chimaerin N94H Δ303-305 displays a more particulate, punctate

appearance, concentrating around the nucleus but which also extends along the neurite structures absent in α2 chimaerin N94H transfection.

The lack of morphological effect from over-expression of wild type α2 chimaerin on neuritogenesis in these cells (Fig 3.7A) is again consistent with lack of Rac GAP activity. There was however a small but significant increase in the number of round cells expressing GFP-α2 chimaerin compared with GAP inactive GFP-α2 chimaerin expressing cells ($p<0.05$), potentially implying partial GAP activity of the expressed wild type α2 chimaerin.

3.B.4 GAP inactive α2 chimaerin expression induces neurite outgrowth in N1E-115 neuroblastoma cells

α2 chimaerin GAP activity inhibits outgrowth and induces collapse of neuroblastoma cell neurites. It was thus intriguing if inhibition of endogenous α2 chimaerin is sufficient to induce neurite outgrowth in these cells. To investigate this, α2 chimaerin GAP inactive cDNAs were transfected, which could act as dominant negative constructs *in vitro* (Ahmed et al., 1994). N1E-115 neuroblastoma cells were transfected with GFP fused constructs, cultured in 5% FCS containing media and morphologically examined.

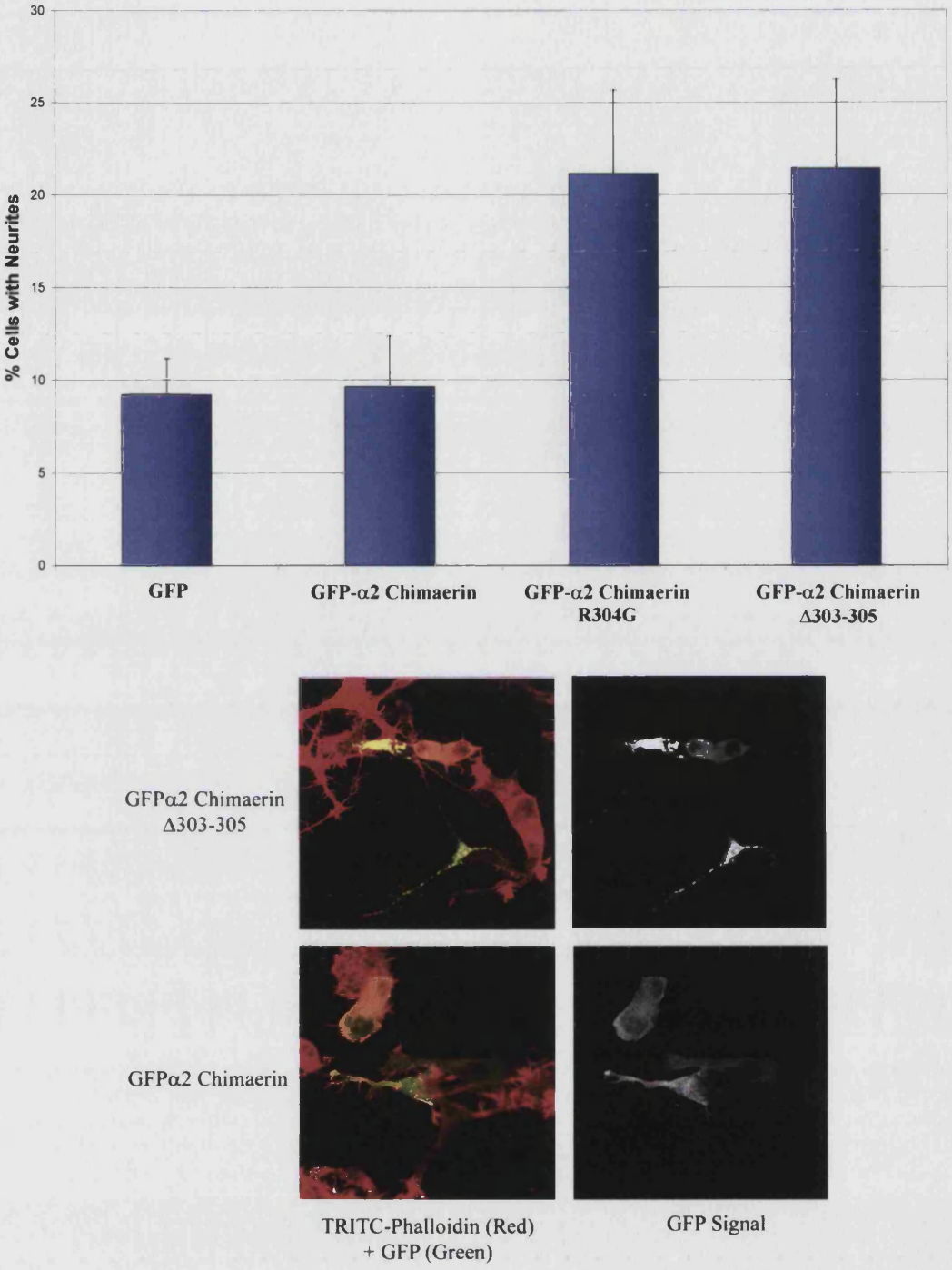


Fig. 3.8 GAP inactive α2 chimaerin expression induces neuritogenesis of N1E-115 neuroblastoma cells. N1E-115 neuroblastoma cells, on poly-L-Lysine, were transiently transfected with GFP fused α2 chimaerin constructs, cultured in 5% FCS containing media for 16hrs and fixed/stained with TRITC-phalloidin. Transfected cells with processes >2 cell body diameters were counted as neurite bearing (error bars indicate SDs for three separate experiments)

Neurite formation in N1E-115 cells is a Rac/Cdc42 dependent phenomenon, which the Rho acting antagonistically serves to inhibit. Culturing cells in 5% FCS containing media inhibits neurite formation, with less than 10% neuroblastoma cells bearing neurites (Fig. 3.8). Over-expression of α2 chimaerin did not significantly alter this ($p>0.1$), again consistent with an inactive state. However the expression of GAP inactive α2 chimaerin constructs lead to significant neurite formation ($p<0.01$), with greater than 20% cells bearing neurites. The expression of the GFP-α2 chimaerin Δ303-305 mutant tended towards aggregated protein distribution, similar to that seen in Cos-7 cells (Fig. 3.1). These mutant constructs potentially act by competitive inhibition of endogenous α2 chimaerin GAP activity. These results therefore possibly imply serum containing factors serve to inhibit neuritogenesis of N1E-115 neuroblastoma cells, in part through inactivation of Rac signalling, via activation of α2 chimaerin GAP activity.

However GAP proteins, including α1 chimaerin, have been shown to promote Rac/Cdc42 effector morphology independent of their GAP activity (Kozma et al., 1996). Thus the observed neurite outgrowth could be an effector/adaptor function of α2 chimaerin. It is of interest to note the two α2 chimaerin GAP inactive mutants induce neurite formation to similar extents despite quite different subcellular distributions (Fig. 3.1).

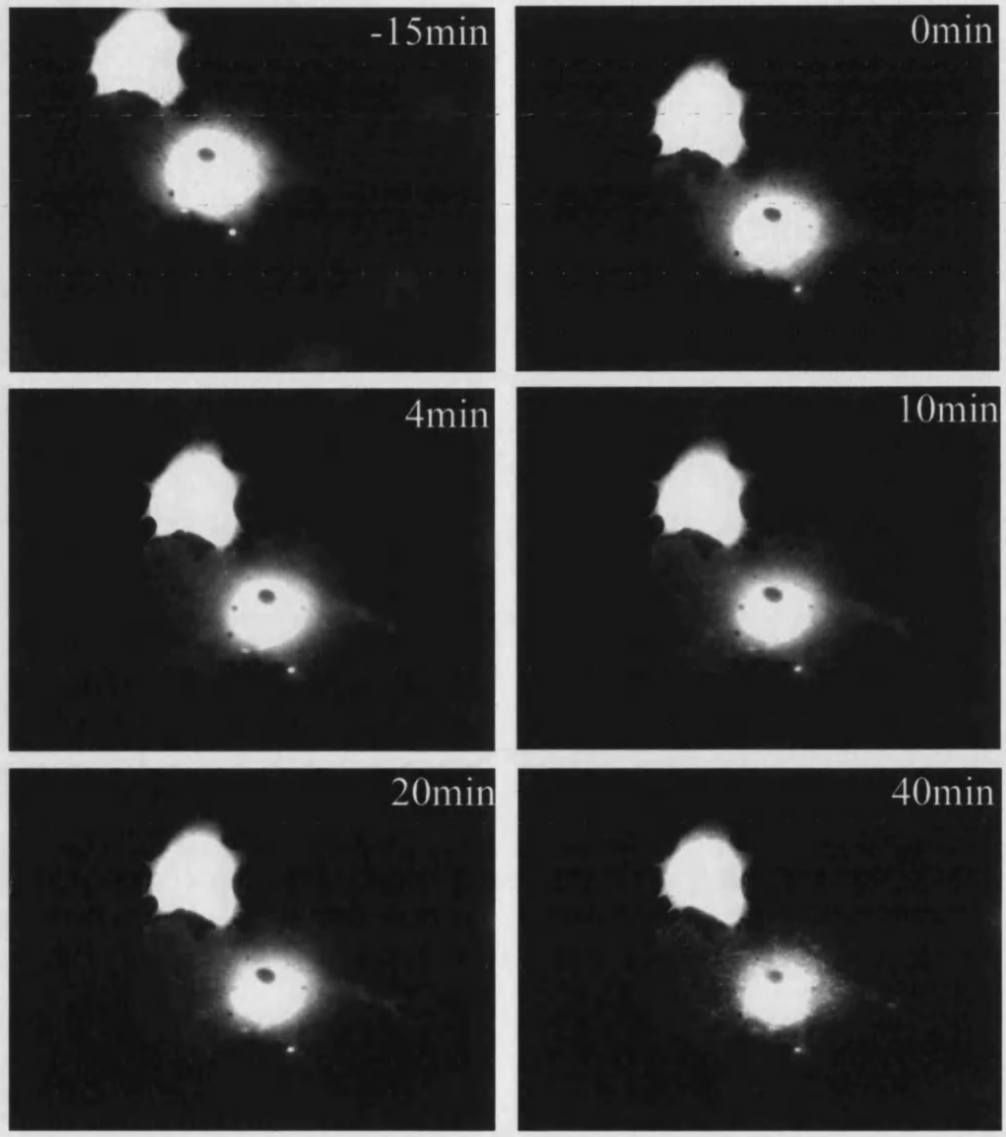
3.C PMA Activates α2 Chimaerin GAP Activity

3.C.1 PMA alters α2 chimaerin distribution in Cos-7 cells

The translocation of α2 chimaerin in neuroblastoma cells, in response to PMA was difficult to visualise due to the fast retraction/collapse response of these cells. In live cell fluorescence imaging, the associated technical problems with these cells are

increased. Therefore the Cos-7 cell line was used for live fluorescent imaging of α2 chimaerin, as the more adhesive, flattened nature of these cells made imaging easier.

(i) GFP α2 Chimaerin



(ii) GFP

Fig. 3.9 PMA translocates α2 chimaerin in Cos-7 cells.

Cos-7 cells seeded onto glass dishes were transiently transfected with GFP-α2 chimaerin (i) or GFP (ii) constructs and cultured in 10% serum containing media. Live cell fluorescence was monitored for 15min, before treating with 10 μM PMA at time 0 and continued thereafter.

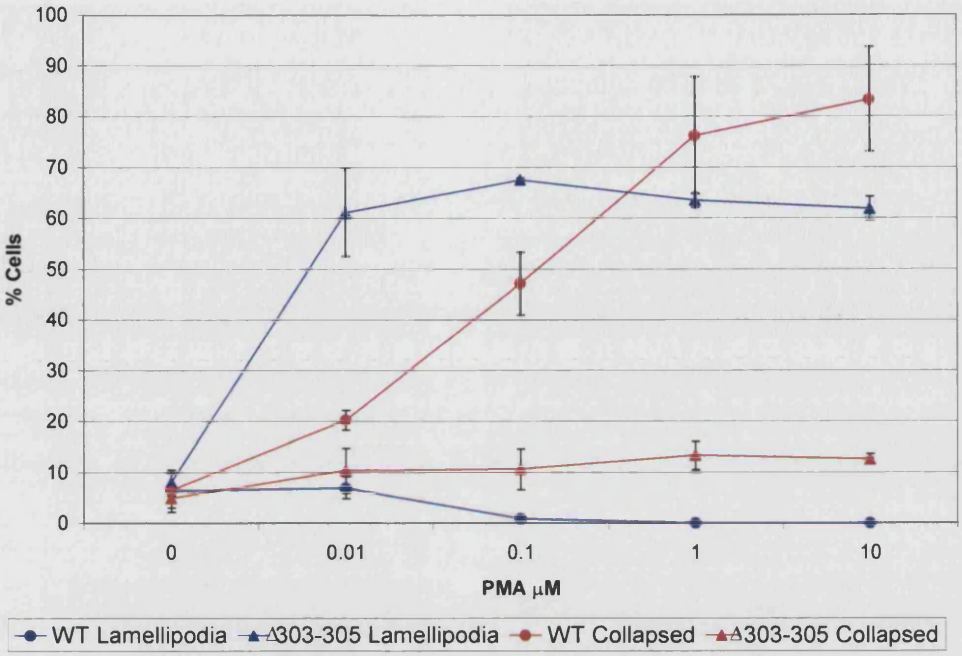
High fluorescence was observed in the cell body due to cell shape, saturating detection and making observations surrounding the nucleus difficult. PMA treatment had very little effect on the distribution of GFP (Fig. 3.9ii), while GFPα2 chimaerin distribution was significantly altered (Fig. 3.9i). GFP α2 chimaerin was uniform throughout the cytoplasm with diffuse fluorescence. Within a few minutes of PMA treatment, GFP α2 chimaerin was shown to aggregate within the cytoplasm with a punctate accumulation, while no plasma membrane localisation was observed. This could represent a redistribution to inner membranes and vesicles in response to PMA in Cos-7 cells.

Imaging using UV radiation caused considerable retraction of GFP expressing cells in the presence or absence of PMA, but interestingly not of GFP-α2 chimaerin expressing cells. However in non-monitored cells PMA induced dramatic collapse of Cos-7 cells expressing GFP-α2 chimaerin but not those expressing GFP (data not shown). The mechanisms of this remain unclear but for morphological analysis, fixed cell studies were carried out.

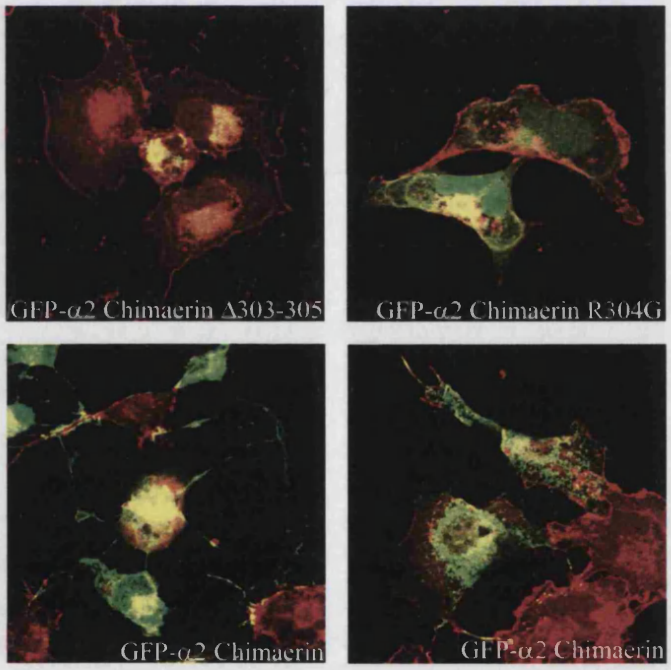
3.C.2 α2 chimaerin inhibits PMA induced lamellipodia in Cos-7 cells

PMA treatment of Swiss 3T3 fibroblasts has been shown to induce lamellipodia, a Rac dependent phenomenon (Ridley et al., 1992), and PMA treatment translocates α2 chimaerin (Fig. 3.9). To investigate the phenotypic effects of PMA on α2 chimaerin expressing Cos-7 cells, cells seeded on glass coverslips were transiently transfected with GFP-α2 chimaerin (WT) and GAP inactive GFP-α2 chimaerin Δ303-305 and treated with a concentration gradient of PMA for 1hr before fixing and staining the cells. Morphological analysis was then undertaken.

A



B



10μM PMA-treated Cos-7 cells

Fig. 3.10 α2 chimaerin Inhibits PMA Induced lamellipodia in Cos-7 cells.

Cos-7 cells seeded on glass coverslips were transfected with wild type chimaerin (circles)or GAP inactive α2-chimaerin (triangles) and cultured in 10% serum containing media. After treatment with PMA for 1 hr, cells were fixed and stained with TRITC phalloidin. Cells with prominent lamellipodia (blue) or having collapsed (red) were counted (A) (error bars indicate +/- SDs from three separate experiments) and imaged (B)

PMA induced prominent lamellipodia in non transfected Cos-7 cells and cell expressing GAP inactive α2 chimaerin Δ303-305 (Fig. 3.10 A+B). This phenotype was inhibited by the exogenous expression of wild type α2 chimaerin, where PMA induced significant cellular collapse. The inhibition of lamellipodia formation and collapse of cells expressing α2 chimaerin upon PMA treatment is thus a direct result of GAP activity. PMA has been shown to translocate α2 chimaerin to lipid environments (Fig. 3.2) and this appears to stimulate GAP activity resulting in a global loss of Rac activity, which under these conditions, results in cellular collapse. Cells were cultured in the presence of 10% FCS which has been shown to activate Rho signalling, thus the removal of the antagonistic Rac could lead to increased actin/myosin contraction. These results again imply α2 chimaerin exists in an inactive state in the cytosol, requiring lipid association for GAP activation, which results in cell collapse.

In a minority number of cells a particulate accumulation of α2 chimaerin was observed in the absence of collapse which was also apparent during live cell imaging (Fig.3.9). This particulate appearance of α2 chimaerin may be through vesicle association or it could be a result of aggregation/insolubilisation of α2 chimaerin, potentially through oligomerisation, which may result in inactive complexes.

3.D Summary of Results I

Phorbol ester treatment has now been shown to translocate α2 chimaerin to membrane and cytoskeletal fractions. Phorbol ester/DAG binding to the C1 domain in some way promotes lipid interactions, whether, like PKC it allows a contiguous hydrophobic surface for insertion, or whether it is a drastic conformational change remains unclear. The target membrane translocation appear cell specific. Lipid binding results in

activation of α2 chimaerin GAP activity which is under the control of the C1 domain via DAG association.

As in neuronal growth cones, PMA treatment causes neurite collapse/retraction of N1E-115 neuroblastoma cells, which can now in part be attributed to α2 chimaerin as well as PKC. α2 chimaerin GAP activity appears to inhibit neuritogenesis and cause neurite retraction in these cells. A mutant disrupting the inactive conformation (N94H), allowing lipid association and GAP activation, inhibits neurite formation while the expression of a GAP inactive α2 chimaerin constructs is sufficient to stimulate neurite formation, through potential inhibition of endogenous α2 chimaerin activity. In Cos-7 fibroblasts, treatment with PMA causes lamellipodia formation but with the exogenous expression of α2 chimaerin, results in cellular collapse in response to PMA.

The differences between α1 and α2 chimaerin subcellular distribution have been attributed to an N-terminal variance, including an SH2 domain in α2 chimaerin (Hall et al., 2001). Influences of the SH2 domain presumably inhibit lipid interactions in some manner, resulting in the cytosolic localisation of α2 chimaerin. N94H mutation in the SH2 domain results in membrane association and GAP activation. These results led to the postulation of a model for α2 chimaerin activation. α2 chimaerin exists in an auto-inhibited state in the cytosol, possibly through association of the SH2 domain with the GAP domain. This closed conformation inhibits lipid interactions as well as GAP activity. This potential intramolecular interaction is likely to be phosphotyrosine independent as mutations in the SH2 domain, which knock out phosphotyrosine binding (R56L and R73L), do not affect cellular distribution (Hall et al., 2001).

Chapter Four

Results II

4. α2 Chimaerin and p35/Cdk5

The proposed model of an auto inhibited conformation of α2 chimaerin out-lined in Results I would allow for tight regulation of GAP activity. Phorbol ester association and subsequent lipid interactions have been proposed to increase α2 chimaerin GAP activity *in vivo*. DAGs are themselves tightly regulated and it is likely specific DAGs, would vary in affinity for the C1 domains of chimaerins, enhancing targeting of GAP activity (Caloca et al., 2001). Specificity in phospholipid binding is also observed with acidic phospholipids such as phosphatidylserine preferred (Ahmed et al., 1993).

Protein-protein interactions could also conceivably serve to regulate GAP activity and location. As with PKC, it is likely proteins exist that interact with the active state, the inactive state or that serve as substrates for α2 chimaerin. Rac binding and GAP activity are distinguishable properties (Ahmed et al., 1994) leading to the postulation that chimaerins could also serve as Rho GTPase effectors, through adaptor functions. Hence protein interactions of α2 chimaerin are of great interest.

In an attempt to identify novel interactors for the neuronal specific activator of Cdk5, p35, Dr Robert Qi undertook a yeast two hybrid screen and isolated α chimaerin as a possible candidate (Qi et al., 2004).

4.A.1 P35 interacts with α2 chimaerin C-terminus

The yeast two hybrid screen identified the C-terminal 98 residues of α chimaerin responsible for the interaction with p35 (Qi et al., 2004). Because of their similar developmental patterns of expression, α2 chimaerin may be the more likely *in vivo* interacting isoform. To confirm an *in vivo* interaction in mammalian cells and identify the region of α2 chimaerin involved, immunoprecipitations from Cos-7 cells over-expressing fragments of α2 chimaerin and full length p35 were carried out.

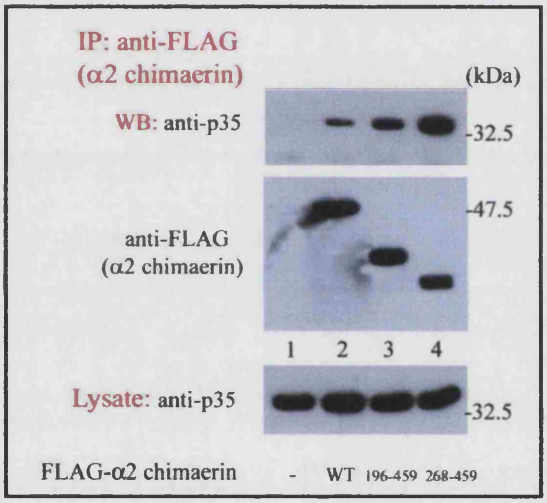


Fig. 4.1 P35 interacts with α2 chimaerin C-terminus in transfected Cos-7 cells.
Cos-7 cells were transiently transfected with p35 +/- FLAG-α2 chimaerin constructs. After 16 hrs expression, cells were lysed and immunoprecipitated with anti-FLAG antibody. Samples were analysed by SDS gel electrophoresis and western blotted with indicated antibodies.

These immunoprecipitations (Fig.4.1) confirm an association of p35 with α2 chimaerin *in vivo*, and the C-terminus of α2 chimaerin (residues 268-459), encompassing the GAP domain was sufficient for interaction (Fig.4.1 lane 4). Thus both α1 and α2 chimaerin isoforms are potential *in vivo* binding partners of p35. There was an increase in association of p35 with N-terminal deleted fragments of α2 chimaerin (Fig.4.1). This is consistent with a restricted conformation of α2 chimaerin involving the N-terminus.

4.A.2 α2 Chimaerin is not an *in vitro* p25/Cdk5 substrate

Cdk5 is a neuronal kinase, thus the association of α chimaerins with p35 implicates a possible substrate role of α chimaerins for Cdk5 activity. Cdk5 is a proline directed Ser/Thr kinase and both α chimaerin isoforms possess a minimal Cdk5 consensus site (S/TP) within their C-terminus (SPEL see Fig. 4.13), with an additional site unique to α1 chimaerin N-terminus (^{39}SPKS). To test whether α chimaerin isoforms could serve as potential substrates for Cdk5 activity, *in vitro* kinase assays were undertaken with recombinant p25/Cdk5 and α chimaerin proteins. p25 is an N-terminal truncated form of p35 (Patrick et al., 1999), which still binds α chimaerin (Qi et al., 2004).

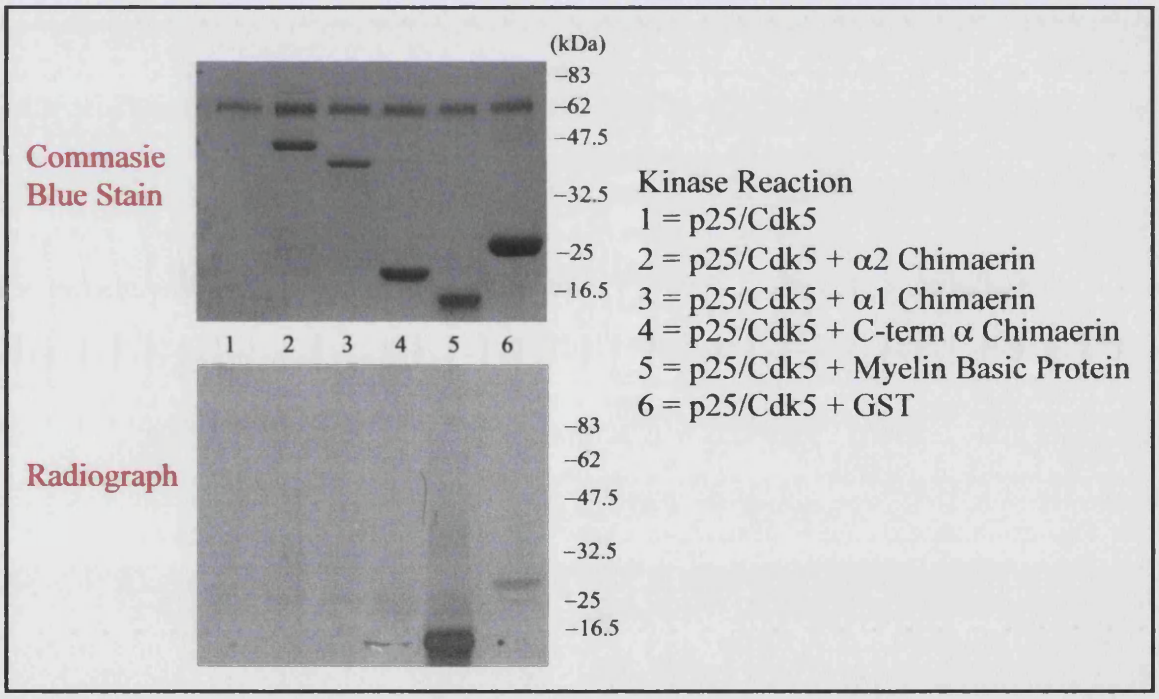


Fig 4.2 p25/Cdk5 *in vitro* kinase assay with recombinant α chimaerin.

5μg of recombinant substrate protein was mixed with 2μl recombinant p25/Cdk5 in a 30 μl Reaction Volume (5μCi ^{32}PγATP, 100 μM ATP, 20 mM MOPS pH 7.4, 5 mM $MgCl_2$) and incubated at 30 ^{0}C for 30min. Samples were analysed by SDS gel electrophoresis and exposed to film.

In vitro kinase assays with recombinant p25/Cdk5 showed that neither α1 or α2 chimaerin served as a substrate for Cdk5 activity under the conditions tested (Fig. 4.2 lane 2 and 3), despite phosphorylation of myelin basic protein (MBP lane 5). To eliminate the possibility of α chimaerin conformational hindrance, a cleaved recombinant peptide fragment comprising α chimaerin C-terminal GAP domain was also utilised, but which remained unphosphorylated (Fig. 4.2 lane 4). Experiments using heat denatured GST-α2 chimaerin also yielded no phosphorylation (data not shown). The favoured consensus for Cdk5 phosphorylation includes a highly basic residue at the +3 position (S/TPXK/R) which is not apparent in either α chimaerin isoform. *In vivo* P25/Cdk5 displays higher activity and reduced specificity compared with p35/Cdk5 (Patrick et al., 1999), therefore this lack of *in vitro* phosphorylation suggests α1 and α2 chimaerins are unlikely to serve as substrates for p35/Cdk5 activity *in vivo.*

These results are in disagreement with Qi et al., 2004, where direct phosphorylation of α1 chimaerin by p25/Cdk5 was observed. This discrepancy is a possible result of the state of recombinant chimaerin proteins used. Recombinant proteins could be treated with phosphatases to investigate whether proteins have been prephosphorylated on any potential Cdk5 site during synthesis and purification.

4.A.3 α2 Chimaerin interacts separately with p35 and Cdk5, but not with the active p35/Cdk5 complex

In vivo, p35 interacts with Cdk5, which it activates. To investigate the relationship of α2 chimaerin with the p35/Cdk5 complex, immunoprecipitation studies from Cos-7 cells over-expressing combinations of these proteins, were carried out.

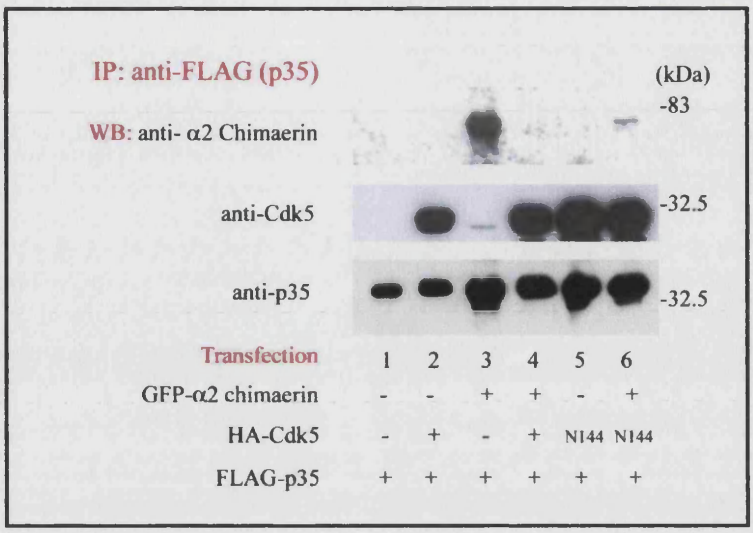


Fig. 4.3 FLAG-p35 immunoprecipitations from transfected Cos-7 cells.
Cos-7 cells were transfected with indicated plasmids and cultured for 16 hrs in 10% FCS containing media before lysis and immunoprecipitation with anti-FLAG antibody. Samples were analysed by SDS gel elecreophoresis and western blotted with indicated antibodies.

FLAG-p35 was immunoprecipitated from Cos-7 cells expressing combinations of p35, Cdk5 and α2 chimaerin. As expected Cdk5 associated with p35 when both proteins are co-expressed (Fig. 4.3 lane 2). α2 chimaerin was also found to associate with p35, when both proteins are expressed in Cos-7 cells (Fig. 4.3 lane 3). When all three proteins were co-expressed in Cos-7 cells, immunoprecipitation of p35 only co-precipitated Cdk5, with no observable α2 chimaerin association (Fig. 4.3 lane 4), implying that there was competitive binding, with much higher binding affinities of p35 for Cdk5 than α2 chimaerin. However α2 chimaerin was co-precipitated with p35 in the presence of the inactive kinase mutant Cdk5N144 (Fig. 4.3 lane 6). In cells Cdk5N144 forms a more stable complex with p35 (Nikolic et al., 1996) as ubiquitin mediated degradation of p35 is initiated by Cdk5 phosphorylation. Since p35/Cdk5 activity has also been found to be detrimental to some cells, in that it induces apoptosis, it is possible that α2 chimaerin may only associate in a hetero-trimer with the inactive and more stable p35/Cdk5N144. Alternatively, this result implies a role for Cdk5 activity in

down regulating α2 chimaerin association with p35. Since α2 chimaerin is not a substrate for Cdk5 (at least under the conditions tested), phosphorylation of p35 by Cdk5 may be responsible for the loss of interaction with α2 chimaerin. It is also possible two separate complexes may be co-precipitated with p35 when the inactive kinase is present (p35/Cdk5N144 and p35/α2 chimaerin).

To further investigate the protein complexes of α2 chimaerin/p35 and Cdk5, immunoprecipitation experiments were also carried out by pulling down either Cdk5 (Fig. 4.4) or α2 chimaerin (Fig. 4.5) with antibody and investigating associated proteins.

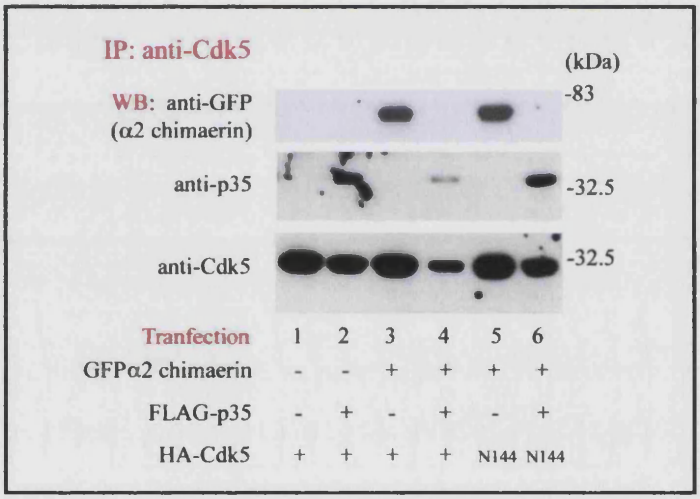


Fig. 4.4 Cdk5 immunoprecipitations from transfected Cos-7 cells.
Cos-7 cells were transfected with indicated plasmids and cultured for 16 hrs before harvesting and immunoprecipitation with agarose conjugated anti-Cdk5 antibody. Samples were analysed by SDS gel electrophoresis and western blotted with indicated antibodies.

Immunoprecipitation of Cdk5 from Cos-7 cells expressing both Cdk5 and p35 resulted in expected p35 pull down (Fig. 4.4 lane 2). Immunoprecipitation of Cdk5 (WT or N144) was also sufficient to co-precipitate α2 chimaerin when co-expressed (Fig. 4.4 lane 3 and 5), suggesting it too interacts directly with α2 chimaerin, although an indirect association cannot be ruled out. However, in the presence of p35, α2 chimaerin association with Cdk5 was abolished (Fig. 4.4 lane 4). With Cdk5

immunoprecipitation, no observable potential trimeric protein complex was evident when α2 chimaerin, p35 and Cdk5N144 or Cdk5 were all over-expressed (Fig. 4.4 lane 4 and6), implying p35 and α2 chimaerin compete for the same binding site on Cdk5.

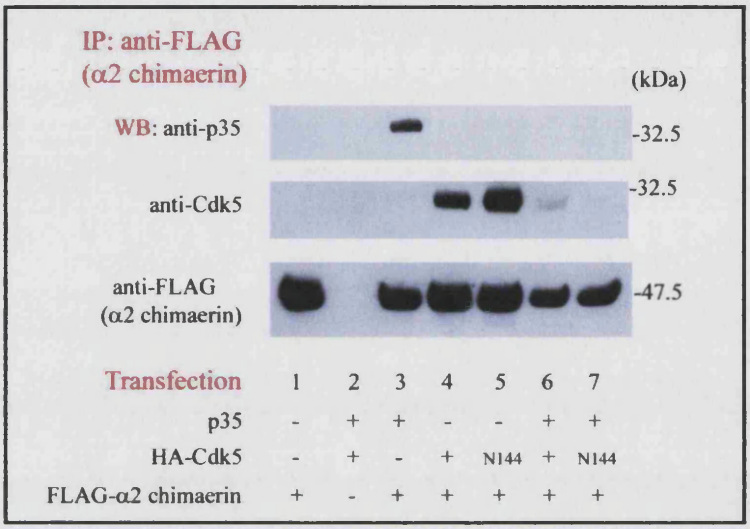


Fig. 4.5 FLAG-α2 chimaerin immunoprecipitation from transfected Cos-7 cells.
Cos-7 cells were transfected with indicated plasmids and cultured for 16 hrs in 10% FCS containing media before harvesting and immunoprecipitation with anti-FLAG antibody. Samples were analysed by SDS gel electrophoresis and western blotted with indicated antibodies.

Immunoprecipitation of FLAG-α2 chimaerin confirmed its association with both p35 and Cdk5 separately (Fig. 4.5 lane 3 and 4). Again when all three proteins were over expressed little or no p35/Cdk5 was co-precipitated with α2 chimaerin, nor was inactive p35/Cdk5N144 (Fig. 4.5 lanes 6 and 7) co-precipitaed with α2 chimaerin.

4.A.4 α2 chimaerin does not regulate p35/Cdk5 *in vitro*

The association of α2 chimaerin with p35 and Cdk5, but its lack of phosphorylation is suggestive of a regulatory role for α2 chimaerin on p35/Cdk5 kinase activity. This was tested with *in vitro* kinase assays, however recombinant GST α2 chimaerin failed to show any significant effect on recombinant p25/Cdk5 kinase activity using MBP as a

control substrate (data not shown). The experiment was repeated using p35/Cdk5 immunoprecipitated from transfected Cos-7 cells as the source of kinase.

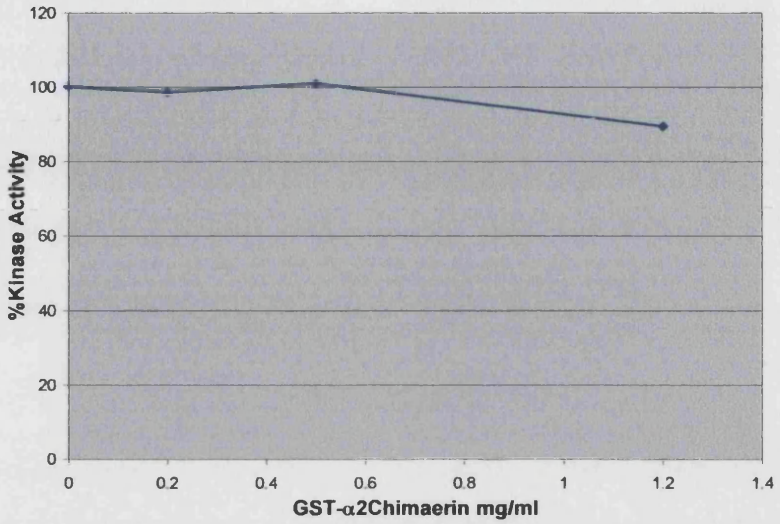


Fig. 4.6 *In vitro* p35/Cdk5 kinase assay in the presence of increasing concentrations of GST α2 chimaerin. Immunoprecipitated Cdk5 from Cos-7 cells expressing FLAG-p35 plus HA-Cdk5 was used in an *in vitro* kinase assay with MBP as a substrate in the increasing presence of recombinant GST-α2 Chimaerin. Activity was expressed as a percentage of the control. A separate experiment gave similar results.

Recombinant GST α2 Chimaerin failed to show any significant effect on p35/Cdk5 kinase activity (Fig. 4.6). However this involved a pre-associated p35/Cdk5 complex and it is quite possible that *in vivo* α2 chimaerin can interfere with the formation of the complex. α2 chimaerin potentially competes for p35 and Cdk5 binding with Cdk5 and p35 respectively, albeit with much lower affinities. Kinase regulation through this mechanism or possibly via down regulation of Rac, a Cdk5 activating protein (Nikolic., et al 1998) is thus still feasible under the appropriate conditions *in vivo.* This lack of *in vitro* regulation of Cdk5 kinase activity by α2 chimaerin is consistent with results of Qi et al., 2004.

4.A.5 PMA promotes α2 chimaerin binding with p35

Because p35 associates with α chimaerin C-terminal GAP domain, p35 could regulate α chimaerin GAP activity. Phorbol ester binding to the C1 domain of α2 chimaerin has been shown to promote lipid association and increase GAP activity, potentially through a conformational change. The relationship between phorbol ester binding and p35 association *in vivo* was therefore investigated. Cos-7 cells were transiently transfected with p35 and α2 chimaerin and treated with PMA before FLAG immunoprecipitations were carried out.

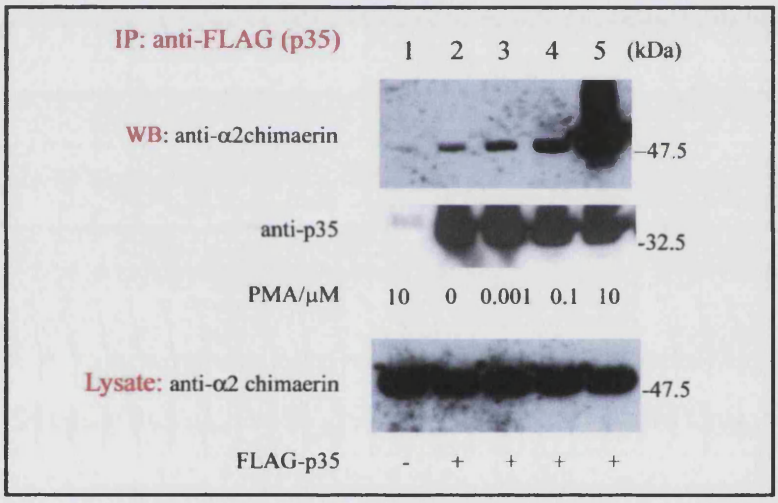


Fig. 4.7 PMA increases *in vivo* binding of p35 and α2 chimaerin.
Cos-7 were transfected with HA-α2 chimaerin +/- FLAG-p35 and cultured for 16 hrs, after which cells were treated with the specified concentration of PMA for 1 hr before lysis and immunoprecipitation with anti-FLAG antibody. Samples were analysed by SDS gel electrophoresis and western blotted with indicated antibodies.

The results clearly showed a concentration dependent increase in the association of α2 chimaerin with p35 in response to PMA (Fig. 4.7). Immunoprecipitations were also carried out with the translocating mutants of α2 chimaerin (Fig. 4.8).

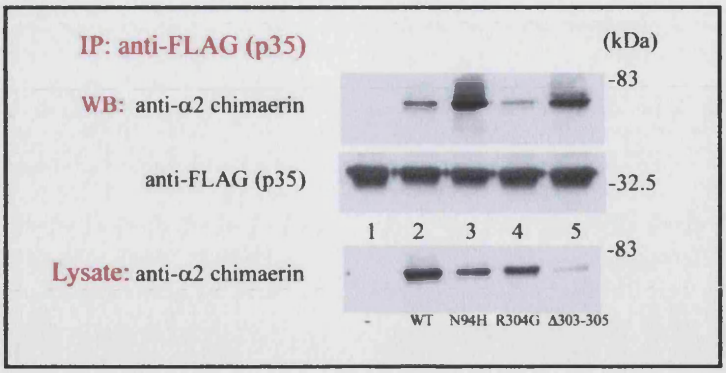


Fig. 4.8 FLAG-p35 immunoprecipitations with α2 chimaerin mutants.

Cos-7 cells were transfected with FLAG-p35 +/- GFP-α2chimaerin or α2chimaerin mutant cDNA constructs and cultured for 16 hrs in 10% FCS containing media before lysing cells and immunoprecipitation with anti-FLAG antibody. Samples were analysed by SDS gel electrophoresis and western blotted with indicated antibodies.

Mutations α2 chimaerin N94H and Δ303-305 showed increased binding to p35 (Fig. 4.8 lane 3 and 5). As with PMA treatment, both these mutations result in translocation of α2 chimaerin from the cytosol to membranous and cytoskeletal fractions (Fig.3.1). P35 is a membrane protein, possessing a myristolation signal at its N-terminus (Patrick et al., 1999). Thus this increase in binding could be a result of increased α2 chimaerin association with membranes, where p35 is localised, or alternatively, these mutations or PMA treatment may alter the conformation of α2 chimaerin, exposing the p35 binding region.

4.A.6 α2 Chimaerin C-terminal GAP domain is sufficient for Cdk5 association

Immunoprecipitation experiments indicated an association of Cdk5 with α2 chimaerin. To determine the region of interaction, FLAG immunoprecipitations from transfected Cos-7 cells were carried out with N-terminal deleted constructs of FLAG-α2 chimaerin (Fig. 4.9).

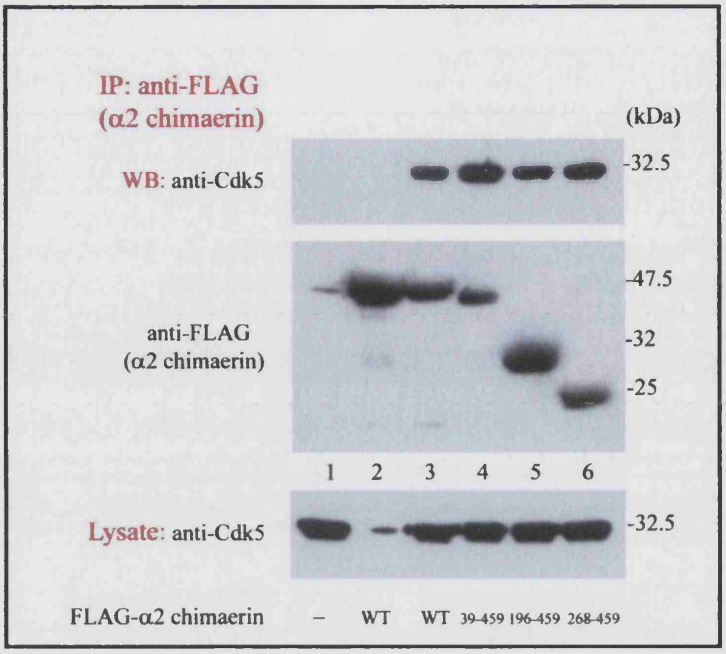


Fig. 4.9 Cdk5 interacts with α2 chimaerin C-terminus in transfected Cos-7 cells.
Cos-7 cells were transfected with HA-Cdk5 (lane1), FLAG-α2 chimaerin (lane 2) or HA-Cdk5 in combination with FLAG-α2 chimaerin constructs as specified (lanes 3-6). 16 hrs post transfection, immunoprecipitations with anti-FLAG antibody were carried out. Samples were analysed by SDS gel electrophoresis and western blotted with indicated antibodies.

The C-terminal GAP domain of α2 chimaerin (residues 268-459) is sufficient for association with Cdk5 (Fig. 4.9 lane 6), as with p35.

To investigate the effect of phorbol ester association on the interaction between Cdk5 and α2-chimaerin, immunoprecipitations of FLAG-α2 chimaerin were carried out after treatment of cells with PMA. Cos-7 cells were transiently transfected and treated with PMA and the association of Cdk5 with FLAG α2 chimaerin analysed (results shown in Fig. 4.10).

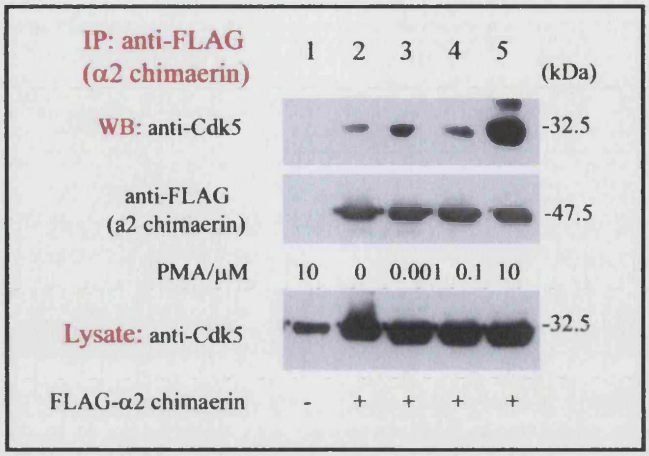


Fig. 4.10 PMA increases α2 chimaerin binding with Cdk5 *in vivo*.

Cos-7 cells were transfected with HA-Cdk5 +/- FLAG-α2 chimaerin. 16 hrs post transfection, cells were treated with indicated PMA concentrations for 1hr before immunoprecipitation with FLAG antibody. Samples were subjected to SDS gel electrophoresis analysis and western blotting with indicated antibodies.

PMA increases the binding of Cdk5 to α2 chimaerin, as with p35 (fig. 4.10). Unlike p35 however, Cdk5 is a cytosolic protein, implying that a conformational change in α2 chimaerin upon PMA binding, and not membrane translocation, is most likely responsible for the increased binding.

4.A.7 PMA increases binding of α2 chimaerin with p35/Cdk5

Since PMA substantially increases the association of α2 chimaerin with p35 and Cdk5 separately (Fig.4.7 and 4.10), it was important to re-evaluate the potential for a trimeric complex of p35/Cdk5 and α2 chimaerin in the presence of PMA.

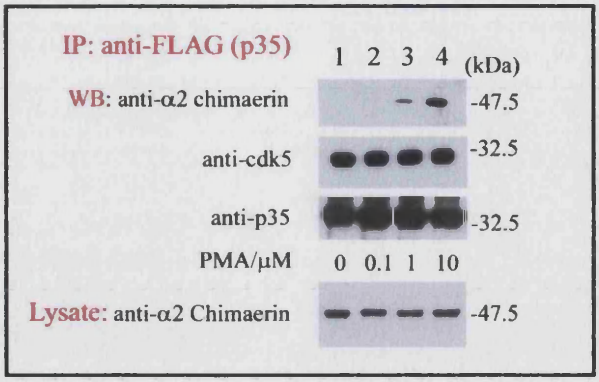


Fig. 4.11 α2 Chimaerin associates with p35/Cdk5 in the presence of PMA.

Cos-7 cells were transfected with FLAG-p35 + HA-Cdk5 + HA-α2 chimaerin cDNAs and cells incubated for 16 hrs to allow protein expression. Cells were treated with indicated PMA concentrations for 1 hr before immunoprecipitation with agarose conjugated anti-FLAG antibody. Samples were analysed by SDS gel electrophoresis and western blotting using the indicated antibodies.

Cos-7 cells were transiently transfected with p35/Cdk5 and α2 chimaerin and subsequently treated with PMA for 1hr and then FLAG-p35 was immunoprecipitated. After treatment with higher concentrations of PMA, α2 chimaerin can associate with p35 in the presence of Cdk5 (Fig. 4.11lane 3 and 4). It is unclear whether a trimeric complex or two separate protein complexes (i.e p35/Cdk5 and p35/α2Chimaerin) are being pulled down. However since the presence of α2 chimaerin in the immunoprecipitate does not appear to alter the level of Cdk5 co-precipiated, it is possible that a trimeric complex can be formed.

In view of this potential trimeric association of p35/Cdk5 kinase complex with α2 chimaerin in the presence of PMA the potential for kinase regulation by α2 chimaerin or for phosphorylation of α2 chimaerin was re-evaluated. To test phosphorylation of α2 chimaerin i*n vitro* kinase assays with recombinant p25/Cdk5 and FLAG immunoprecipitated α2 chimaerin and chimaerin fragments from transfected Cos-7 cells, in the presence of PMA, were undertaken (Fig. 4.12).

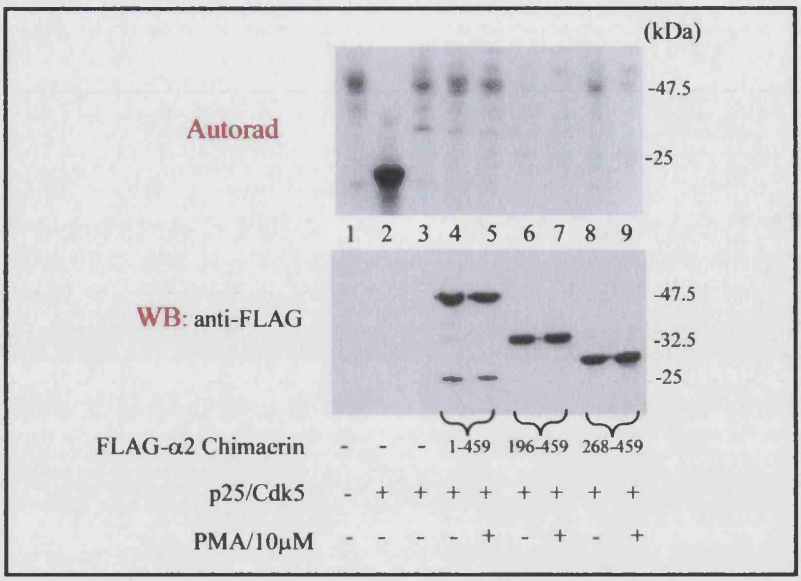


Fig. 4.12 p25/Cdk5 *in vitro* kinase assay with immunoprecipitated FLAG-α2 chimaerin proteins. Cos 7 cells were transiently transfected with various FLAGα2 chimaerin domain constructs and allowed to express for 16 hrs before immunoprecipitations were carried out with FLAG antibody to isolate the sample proteins. Isolated proteins including recombinant MBP (lane 2), were re-suspended in kinase buffer (20 mM MOPS pH7.4, 5 mM $MgCl_2$) with or without PMA for 30 min. *In vitro* kinase assays were then undertaken on these samples, incubating with recombinant p25/Cdk5 30 ^{0}C for 30 min in the presence of ^{32}PγATP.

α2 chimaerin conformation appears crucial for activity. Recombinant proteins isolated from prokaryotic cells potentially lack native conformation and thus isolated mammalian cell expressed proteins are advantageous. The lack of phosphorylation of the various FLAG-α2 chimaerin proteins in the presence or absence of PMA by p25/Cdk5 further suggests a non substrate role of α2 chimaerin for p35/Cdk5 activity (Fig. 4.12).

It was of interest to note a clear 22kDa (~200 residues) protein in the immunoprecipitate containing the FLAG epitope. It is highly likely this represents an N-terminal proteolytic cleavage product of full length α2 chimaerin which was immunoprecipitated using FLAG antibodies (Fig.4.12 lane 4 and 5). A single proteoytic cleavage at this site could possibly release a C-terminal cleavage product

encompassing the C1-GAP domains and could implicate proteolytic cleavage of α2 chimaerin as a further regulatory mechanism.

```
1   maltlfdtde yrppvwksyl yqlqqeaphp rritctceve nrpkyygref hgmisreaad
61  qllivaegsy liresqrqpg tytlalrfgs qtrnfrlyyd gkhfvgekrf esihdlvtdg
121 litlyietka aeyiakmtin piyehvgytt lnrepaykkh mpvlkethde rdstgqdgvs
181 ekrltslvrr atlkeneqip kyekihnfkv htfrgphwce ycanfmwgli aqgvkcadcg
241 lnvhkqcskm vpndckpdlk hvkkvyscdl ttlvkahttk rpmvvdmcir eiesrglnse
301 glyrvsgfsd liedvkmafd rdgekadisv nmyediniit galklyfrdl piplityday
361 pkfiesakim dpdeqletlh ealkllppah cetlrylmah lkrvtlheke nlmnaenlgi
421 vfgptlmrsp eldamaalnd iryqrlvvel liknedilf

RED = SH2
BLUE = CRD
GREEN= GAP BLOCKs CONSTITUTING GAP DOMAIN
```

Fig. 4.13 α2 chimaerin peptide sequence

An alternative approach to investigate α2 chimaerin function with p35/Cdk5 was to transfect p35/Cdk5 and α2 chimaerin into Cos-7 Cells, treat with PMA and isolate the active complex by immunoprecipitation. The addition of ^{32}PγATP could then be used to test for the phosphorylation of α2 chimaerin or the kinase activity of the Cdk5 complex.

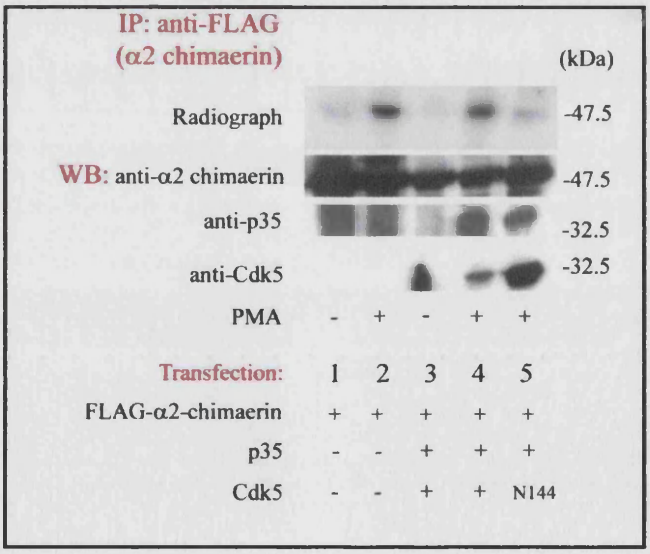


Fig. 4.14 I*n vitro* kinase assay with immunoprecipitated FLAG-α2 chimaerin proteins.

Cos-7 cells were transiently transfected with FLAG-α2 chimaerin + p35 + HA-Cdk5 and allowed to express for 16 hrs before treating +/-PMA lysing and immunoprecipitating with anti-FLAG antibody to isolate the sample proteins. Isolated proteins were re-suspended in kinase buffer (20 mM MOPS pH7.4, 5 mM $MgCl_2$) and incubating at 30 ^{0}C for 30 min in the presence of ^{32}PγATP.

Transfecting Cos-7 cells with FLAG-α2chimaerin and immunoprecipitating with FLAG antibody followed by addition of ^{32}PγATP resulted in no clear phosphorylation of α2 chimaerin or any other proteins (Fig. 4.14 lane 1). However treatment with PMA before FLAG immunoprecipitation did result in phosphorylation of α2 chimaerin upon addition of labelled ATP, but no detection of associated phosphorylated proteins. This suggests the association of α2 chimaerin with a kinase upon PMA treatment, leading to phosphorylation with the addition of ATP. Alternatively PMA activates an associated kinase leading to phosphorylation. The co-expression of p35/Cdk5 with FLAG-α2 chimaerin and treatment with PMA did not increase phosphorylation of α2 chimaerin (lane 4) despite the presence of p35/Cdk5 in the immunoprecipitate. This suggests α2 chimaerin does not serve as a significant downstream target of Cdk5 activity. However the co-expression of p35/Cdk5N144 with FLAG-α2-chimaerin did reduce phosphorylation upon PMA treatment suggesting Cdk5 activity may in some way regulate phosphorylation of α2 chimaerin directly or indirectly.

Repeating this experiment but immunoprecipitating with anti-Cdk5 antibody and monitoring phosphorylation of p35 and exogenously added MBP also indicated that the presence of α2 chimaerin did not appear to regulate p35/Cdk5 activity *in vitro* (data not shown)

4.A.8 p35 or CRMP-2 co-expression does not regulate α2 chimaerin induced morphology

Lipid association stimulates α chimaerin GAP activity and it was of interest to determine whether protein interactions of α2 chimaerin could regulate this. Collapsin Response Mediator Protein-2 (CRMP-2) was purified from rat brain extract (Teo Thesis

1994) and is shown here (Results III) to be an *in vivo* interactor of α2 chimaerin SH2 domain. CRMP-2 and p25/Cdk5 did not regulate α2 chimaerin GAP activity *in vitro* (Teo Thesis 1994, Qi et al., 2004) and so an *in vivo* assay was used to investigate functional correlates of the interaction in cells.

α2 chimaerin N94H appears to be a GAP-active mutant of α2 chimaerin, which inhibits neurite induction of N1E-115 neuroblastoma cells, upon serum withdrawal (Fig.3.7) and interacts strongly with both p35 and CRMP-2 (Fig. 4.8 and Fig. 5.3). This active mutant of α2 chimaerin was therefore used to investigate whether protein interactions with either p35 or CRMP2 could alter α2 chimaerin GAP regulated neuroblastoma cell morphology.

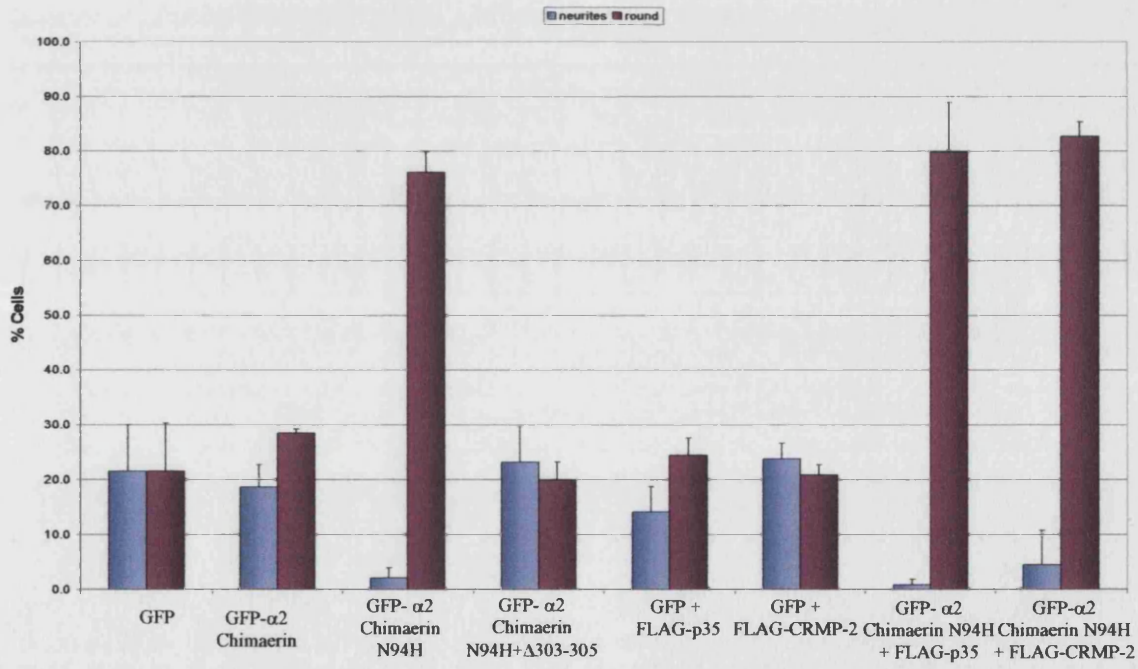


Fig. 4.15 Co-expression of CRMP-2 or p35 with α2 chimaerin N94H in N1E-115 neuroblastoma cells. N1E-115 neuroblastoma cells on poly-L-Lysine were transiently transfected with indicated plasmids before culturing for 16hrs with 1% FCS containing media. Cells were then fixed and stained with TRITC- phalloidin and the number of transfected cells bearing processes>2 cell body diameters were counted as neurite bearing (blue columns) and round cells (red columns) were also counted (error bars indicate SDs from three separate experiments)

Culturing N1E-115 neuroblastoma cells in low serum containing media results in neuritogenesis, which α2 chimaerin GAP activity was found to inhibit (Fig. 3.7). Over-expression of CRMP-2 alone had no effect on neuritogenesis under low serum conditions (Fig. 4.15). Over expression of p35 did appear to reduce neuritogenesis, but this was not statistically significantly ($p>0.1$) (Fig. 4.15). α2 chimaerin N94H inhibits neuritogenesis and interacts strongly with p35 and CRMP-2. The co-expression of either p35 or CRMP-2 with α2 chimaerin N94H did not affect inhibition of neurite formation suggesting GAP activity was unaffected.

In Cos-7 cells α2 chimaerin inhibited PMA induced lamellipodia formation (Fig. 3.10), and PMA also increased α2 chimaerin association with p35 and with CRMP-2 (Fig.4.7.and Fig.5.2). Co-expression in Cos-7 cells of α2 chimaerin with either p35 or CRMP-2 and subsequent PMA treatment stimulates both α2 chimaerin GAP activity and target protein interactions and therefore provides a system for analysis of the effect of partner proteins on α2 chimaerin biological activity.

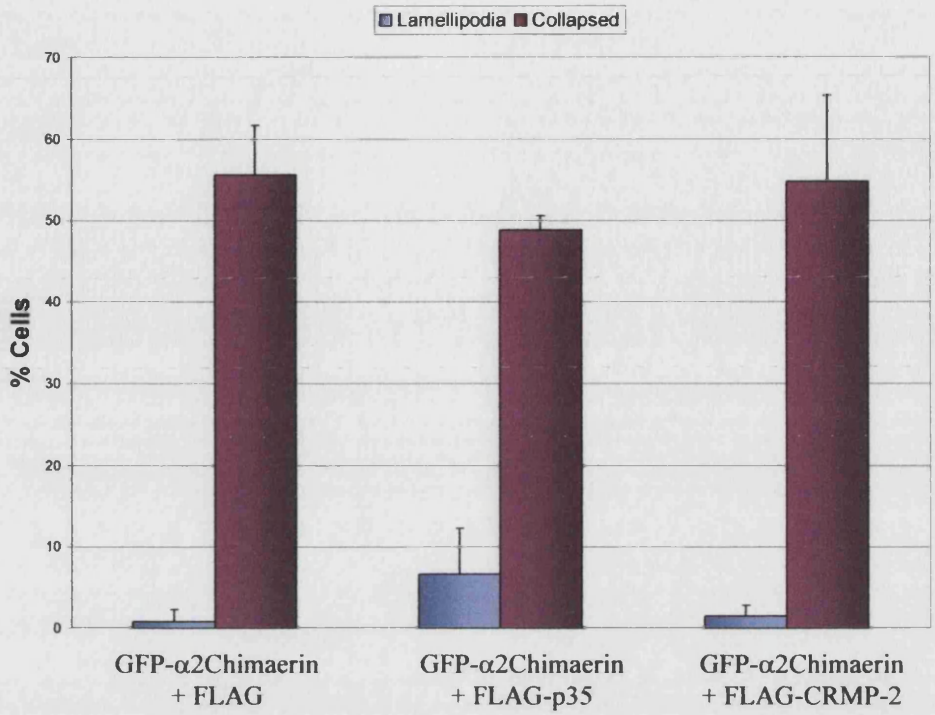


Fig. 4.16 PMA treatment of Cos-7 cells co-expressing CRMP-2 or p35 with α2 chimaerin. Cos-7 cells were transfected with GFP-α2-chimaerin plus FLAG-p35 or FLAG-CRMP-2 and proteins allowed to express for 16 hrs. Cells were then treated with 100 nM PMA for 1hr before fixing and staining with TRITC phalloidin. The number of transfected cells possessing prominent lamellipodia (blue columns) or having undergone collapse (red columns) were counted. Error bars indicate SDs from three separate experiments.

Cos-7 cells expressing α2 chimaerin do not form lamellipodia upon PMA treatment as a direct result of α2 chimaerin GAP activity (Fig.3.10). There was no significant change in the number of cells possessing lamellipodia or having collapsed in cells co-expressing CRMP-2 and α2 chimaerin (Fig. 4.16). Thus the interaction of CRMP-2 with α2 chimaerin does not appear to inhibit PMA-induced α2 chimaerin GAP activation. The co-expression of p35 with α2 chimaerin also had no significant morphological effect on PMA stimulated Cos-7 cells compared with α2 chimaerin expressing cells (Fig.4.16) , indicating p35 association with α2 chimaerin also does not inhibit GAP activity. However these results can not rule out that p35 and CRMP-2 protein interactions regulate α2 chimaerin GAP activity in permissive cell types.

4.A.9 p35 expression in neuroblastoma cells disassociates α2 chimaerin from the membrane fraction

The above studies did not detect any significant regulation of α2 chimaerin activity by association with CRMP-2 or p35. Since α2 chimaerin is active when translocated to membranes, the effect of p35 association on α2 chimaerin localisation was evaluated. α2.10 neuroblastoma cells, permanently expressing α2 chimaerin, were transiently transfected with FLAG-p35 and incubated for increasing time periods to allow p35 accumulation. Cells were then harvested and fractionated and the levels of a2 chimaerin in the membrane fraction (1% Triton soluble) examined (Fig. 4.17).

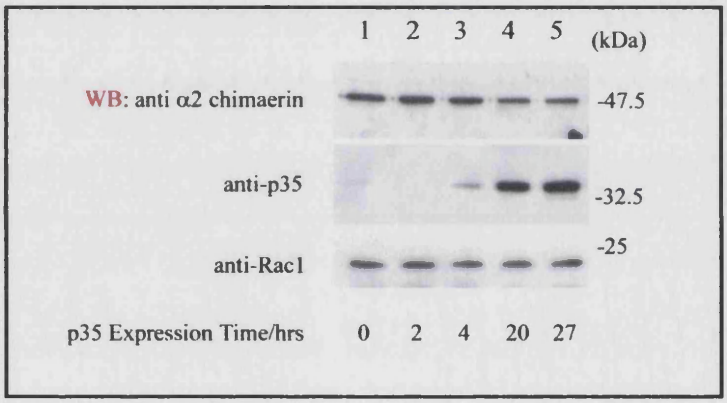


Fig. 4.17 α2.10 neuroblastoma cells 1% Triton soluble fraction with p35 expression.
α2.10 neurobastoma cells were transiently transfected with FLAG-p35 and allowed to express proteins for indicated hours. Cells were fractionated and 25 μg of 1% Triton-x-100 soluble fraction was analysed by SDS gel electrophoresis and western blotted with indicated antibodies.

P35 was detectable 4 hrs post transfection and showed increased accumulation in the membrane fraction with time, up to 27hours (later time not shown) upon transient transfection (Fig. 4.17). At the same time the levels of α2 chimaerin in this fraction appeared to decrease, while endogenous Rac protein levels remained constant. Thus it would appear p35 expression in these cells removes α2 chimaerin from the 1% Triton soluble fraction. This fraction largely represents membranes and thus p35 binding to

α2 chimaerin potentially disassociates α2 chimaerin from the lipid membranes, although an indirect consequence of p35 signalling can not be ruled out. A corresponding increase in α2 chimaerin levels in the other fraction could not be detected (data not shown). This may be due to the abundance of α2 chimaerin in the cytosol or alternatively p35 expression could lead to α2 chimaerin degradation

4.A.10 Cdk5 kinase activity is not required for neuritogenesis of N1E-115 neuroblastoma cells

Cdk5 has been implicated in both neurite outgrowth and growth cone collapse. In human SH-SY5Y neuroblastoma cells, p35 expression is enhanced upon laminin engagement and neuronal differentiation, promoting outgrowth with associated NF-H chain phosphorylation (Li et al., 2002). N1E-115 neuroblastoma cells differentiate upon serum withdrawal and this is enhanced upon laminin engagement. To examine endogenous proteins levels during N1E-115 neuroblastoma cell differentiation, cells were seeded on to laminin and cultured in 10% serum or serum free containing media for 16 hrs before western blotting.

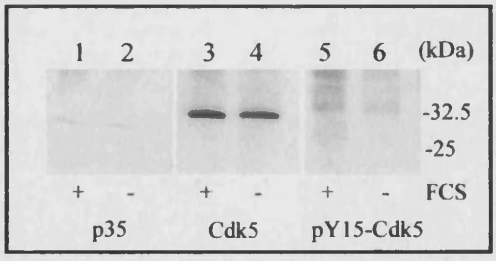


Fig. 4.18 Endogenous proteins of N1E-115 neuroblastoma cells.
N1E-115 neuroblastoma cells on laminin were cultured in the presence or absence of 10% FCS containing media for 16hrs before harvesting. Western blotting of whole cell lysates (40 μg protein) with indicated antibodies was carried out.

Cdk5 was easily detectable in N1E-115 cells and its levels were unaffected by serum starvation and differentiation (Fig. 4.18 lanes 3 + 4). Endogenous p35 protein was undetectable in N1E-115 cells with numerous commercial antibodies, even upon differentiation (Fig. 4.18 lanes 1 + 2). Tyr^{15} phosphorylation of Cdk5 has been shown to promote kinase activity and is thus a further indicator of activity. No Tyr^{15} phosphorylation of Cdk5 was detected using the phosphospecific pY15 antibody. These results imply a lack of p35 expression in N1E-115 neuroblastoma cells and thus a lack of involvement of p35/Cdk5 activity in early differentiation and neuritogenesis.

To further investigate a role of Cdk5 kinase in N1E-115 neuroblastoma cell differentiation, cells were transiently transfected with inactive Cdk5N144 and cultured in low serum (Fig. 4.18).

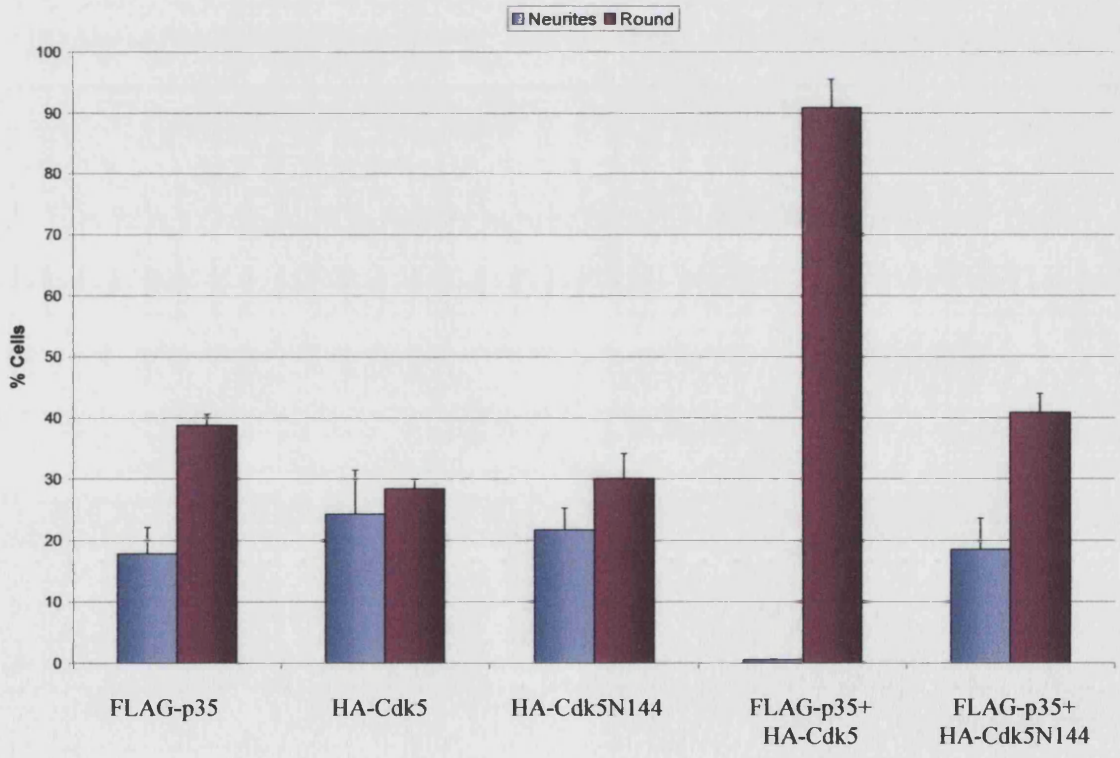


Fig. 4.19 Cdk5 activity in differentiating N1E-115 neuroblastoma cells.

N1E-115 neuroblastoma cells on Poly(L)lysine were transfected with indicated constructs and cultured in low serum (1% FCS) containing media for 16 hrs, before fixing and staining. Transfected cell morphology was then analysed. Error bars indicate SDs for 3 separate experiments.

Over-expression of either Cdk5 or Cdk5N144 had no effect on neurite outgrowth with ~20% of cells bearing neurites as with non transfected cells (Fig. 4.19 and Fig 3.6). Cdk5N144 is a kinase inactive mutant and binds p35 with 2-3x the affinity of native protein, acting as a dominant negative construct. These results imply Cdk5 kinase activity is not required for serum withdrawal induced neuritogenesis of N1E-115 neuroblastoma cells. Over-expression of these proteins also did not lead to any significant neuritogenesis when cells were cultured in 5% serum (data not shown). However, co-expression of p35 and Cdk5 inhibited N1E-115 neuritogenesis induced by low serum, with resulting round neuroblastoma cells (Fig.4.19). This is a direct affect of Cdk5 kinase activity as shown by the transfection of p35 + Cdk5N144, which was without effect. Cdk5 activity has been linked to neuronal death which has not been excluded here.

4.B Summary of Results II

α2 chimaerin can associate with both lipids and proteins. Protein interactions could be undertaken for adaptor function, or may serve to regulate localisation or GAP activity. Here α2 chimaerin was found to interact with the activator of Cdk5, p35 and also with Cdk5 itself. The GAP domain of α2 chimaerin was sufficient for these interactions, with the presence of phorbol ester increasing binding. Mutants of α2 chimaerin which showed increased lipid association also displayed increased binding to p35, suggesting a proposed closed conformation of α2 chimaerin inhibits p35 and Cdk5 interactions as well as lipid associations and GAP activity. A complex relationship between α2 chimaerin and p35/Cdk5 may exist with potential competitive binding and phosphorylation regulated interactions.

α2 chimaerin did not appear to serve as a substrate for, or regulate p35/Cdk5 activity under the conditions tested. Neither did the interactions of p35 and CRMP-2 appear to alter stimulated GAP activity of α2 chimaerin. However results suggest p35 association with α2 chimaerin may inhibit lipid membrane association and possibly inhibit GAP activation to some degree. Proteolytic cleavage of α2 chimaerin was also observed in a cellular system, which may serve as a further regulatory mechanism, although not investigated here.

Cdk5 activity was found not to be required for neuritogenesis of N1E-115 neuroblastoma cells, and excess activity resulting from co-expression of p35 and Cdk5 was sufficient to inhibit neurite formation.

Chapter Five

Results III

5. α2 Chimaerin SH2 Domain and Associated Phosphorylation Pathways

The N-terminus of α2 chimaerin, encompassing the putative SH2 domain, appears to be responsible for its predominant cytosolic localisation, where α2 chimaerin may reside in an autoinhibited conformation. DAG binding to the C1 domain, promotes lipid insertion and GAP activation, although the precise mechanisms remain unclear. The SH2 domain of α2 chimaerin could play a part in targeting of GAP activity. Relief of the autoinhibited structure, upon DAG association, could allow intermolecular interactions regulated by tyrosine phosphorylation, locally regulating α2 chimaerin distribution on the membrane. A high affinity receptor for the SH2 domain may alternatively be sufficient to "open up" the α2 chimaerin structure, recruiting to target membrane/non membrane receptors.

α chimaerins have also been shown to display possible effector functions (Kozma et al., 1996). The SH2 domain may be vital for any adaptor function, recruiting α2 chimaerin to tyrosine kinase signalling events. Thus targets of the SH2 domain are of considerable interest. Previous work to identify such targets utilised a labelled α2 chimaerin SH2 domain to probe rat brain extract, and resulted in the isolation of a neuronal phosphoprotein subsequently identified as CRMP-2 (Teo Thesis 1994, Minturn et al., 1995).

Phosphorylation is the most well characterised post translational modification of proteins, responsible for dynamic regulation and forms the basis for transient protein interactions. Phosphorylation of GAP proteins has been found to regulate activity and possibly even alter specificity (Minoshima et al., 2003) whilst phosphorylation of microtubule binding protein alters their affinities for microtubules, with

hyperphosphorylation of Tau and CRMP-2 associated with neurodegenerative diseases (see 1.G.4).

5.A. Phosphotyrosine binding is not required for GAP inactive α2 chimaerin induction of neurites

α2 chimaerin has been shown to display possible effector/adaptor functions independent of its GAP activity, promoting neurite formation in N1E-115 neuroblastoma cells (Fig.3.7). If this represents an adaptor function, the role of the SH2 domain and phosphotyrosine association may be of importance. To investigate this a double mutant eliminating phosphotyrosine binding capability and GAP activity (α2chimaerin R73L-R304G) was utilised.

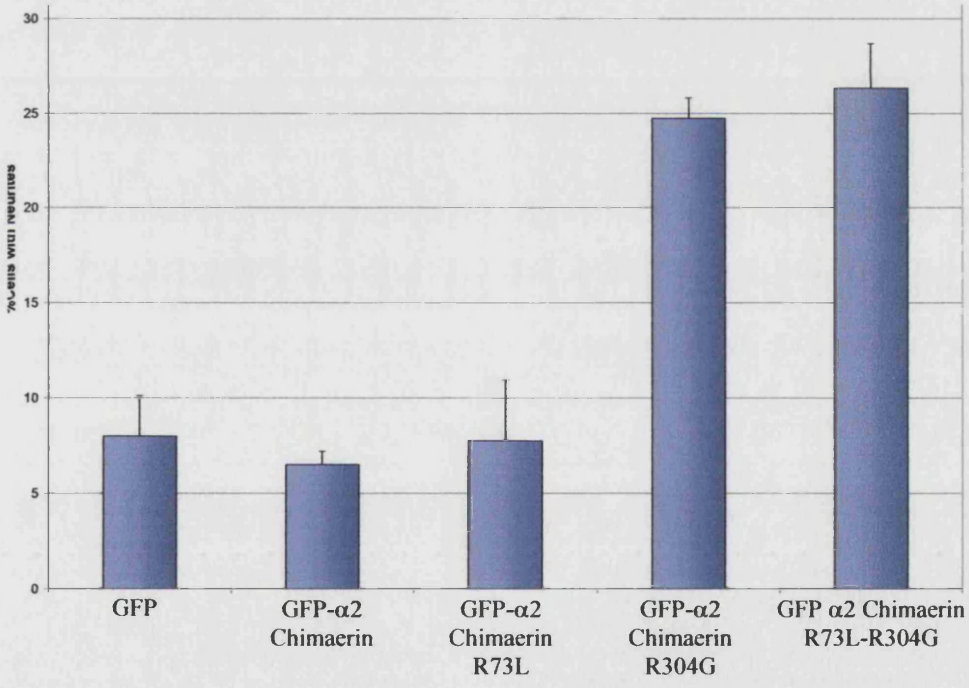


Fig. 5.1 Expression of α2 chimaerin phosphotyrosine binding mutants in neuroblastoma cells. N1E-115 neuroblastoma cells were transiently transfected with GFP constructs and cultured in 5% FCS containing media. 16hrs post transfection, cells were fixed and stained and the number of transfected cells bearing neurites were counted. Error bars indicate SDs for three separate experiments.

Over-expression of α2 chimaerin, or α2 chimaerin R73L, with eliminated phosphotyrosine binding capability, does not induce any significant neuritogenesis of N1E-115 neuroblastoma cells, in 5% serum containing media. GAP inactive α2 chimaerin R304G, does induce significant neurite formation which appears to be independent of phosphotyrosine binding as shown by the double mutant α2chimaerin R73L-R304G. The question of whether this neurite induction is an effector function of α2 chimaerin or whether it is a result of inhibition of endogenous α chimaerins remains unclear. However the absence of an α2 chimaerin R73L effect in serum does not preclude a receptor mediated response to other specific signals.

5.B α2 Chimaerin and CRMP-2

5.B.1 PMA increases CRMP-2 association with α2 chimaerin *in vivo*

Since CRMP-2 is a potential novel interactor of the α2 chimaerin SH2 domain, which has been shown to interact *in vitro* assays (Teo Thesis 1994), it was sought to determine if these proteins could interact *in vivo* in a cellular system. Previous immunoprecipitation studies of α2 chimaerin yielded minimal association of CRMP-2 with α2 chimaerin (Caan Thesis 2000). In view of the effects of α2 chimaerin mutations and PMA it was of interest to determine if this lack of association was due to the proposed closed conformation of α2 chimaerin. PMA activates α2 chimaerin and immunoprecipitations were therefore undertaken in the presence of PMA. Cos-7 cells were transiently transfected with FLAG-CRMP-2 and HA-α2 chimaerin, treated with a range of PMA concentrations and immunoprecipitated with FLAG antibodies. Controls were also set up to confirm PMA was not causing trapping of α2 chimaerin to agarose conjugated FLAG antibodies.

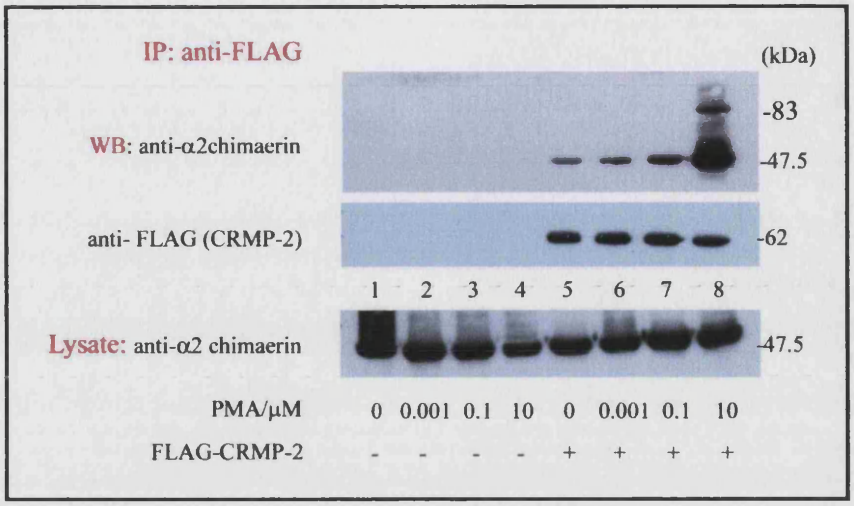


Fig. 5.2 PMA increases *in vivo* binding of CRMP-2 with α2 chimaerin.

Cos 7 cells were transfected with HA-α2 chimaerin alone (lanes 1-4) or in combination with FLAG-CRMP-2 (lanes 5-8). 16hrs post transfection, cells were treated with increasing PMA concentrations for 1hr before lysis and immunoprecipitation with anti-FLAG antibodies. Samples were analyses by SDS gel electrophoresis and western blotted with indicated antibodies.

When FLAG-CRMP-2 was immunoprecipitated from over expressing Cos-7 cells a clear dependence on PMA for the association of α2 chimaerin and CRMP-2 was shown (Fig. 5.2). In the absence of PMA there was little α2 chimaerin association with FLAG-CRMP-2 (Fig. 5.2 lane 5) but in the presence of PMA above 100 nM concentrations, the association was drastically increased (Fig. 5.2 lane 8). Of interest to note is, in this instance, at high PMA concentrations (10 μM) an approximate 90 kDa band was detected by the α2 chimaerin antibody. This could represent a dimer of α2 chimaerin which has not been completely denatured in the SDS gels. This dimerisation event may be explained by the conversion of a potential intramolecular bond to an intermolecular one: In response to PMA, α2 chimaerin is translocated/opened up, and at high concentration when over expressed, α2 chimaerin molecules could dimerise through potential SH2 and GAP domain interactions.

To further investigate α2 chimaerin association with CRMP-2, immunoprecipitations with the translocating α2 chimaerin N94H mutant were also undertaken.

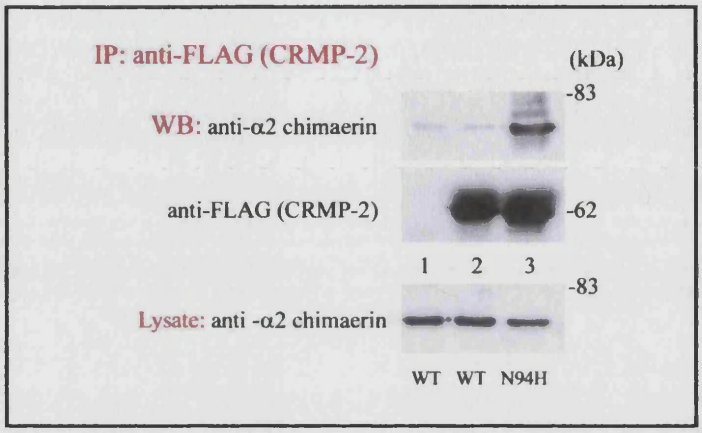


Fig. 5.3 FLAG-CRMP-2 immunoprecipitates with α2 chimaerin N94H mutant.

Cos-7 cells were transfected with FLAG-CRMP-2 +/- GFP-α2chimaerin or GFP-α2chimaerin N94H and cultured for 16 hrs in 10% FCS containing media before lysis and immunoprecipitation with anti-FLAG antibodies. Samples were analysed by SDS gel electrophoresis and western blotted with indicated antibodies

No significant co-precipitation of wild type α2 chimaerin was observed with immunoprecipitated FLAG-CRMP-2 (Fig. 5.3 lane 2). However, α2 chimaerin N94H was able to co-precipitate with CRMP-2 (Fig. 5.3 lane 3). Thus as with p35, an increased association of α2 chimaerin with CRMP-2 was observed with α2 Chimaerin N94H mutant (Fig. 4.8 and 5.3)

5.B.2 α2 chimaerin N-terminal region is required for CRMP-2 association

The detection of CRMP-2 from brain extract using labelled GST-α2 chimaerin SH2 domain (Teo Thesis 1994), indicated a direct interaction with this domain. To confirm this interaction *in vivo* with α2 chimaerin, immunoprecipitates of amino-terminal deleted constructs of α2 chimaerin were carried out in the presence/absence of PMA and tested for the presence of CRMP-2 by western blotting.

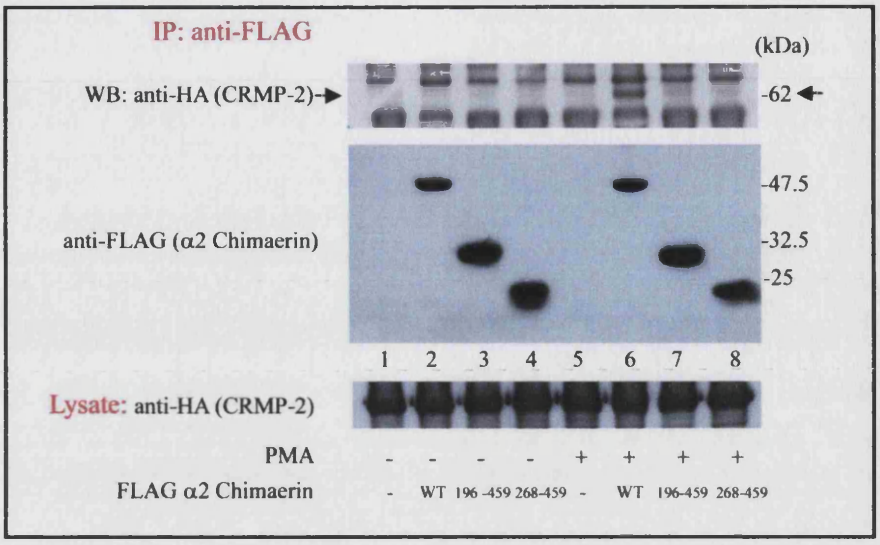


Fig. 5.4 CRMP-2 associates with α2 chimaerin N-terminus.

Cos-7 cells were transfected with HA-CRMP-2 (all lanes) +/- FLAG-α2 chimaerin cDNA constructs (WT, 196-459 and 268-459) and treated with +/- 10 μM PMA for 1hr before lysis and immunoprecipitation with anti-FLAG antibody. Samples were analysed by SDS gel electrophoresiss and western blotted with indicated antibodies.

When FLAG-α2 chimaerin and CRMP-2 were co-expressed in Cos-7 cells, on immunoprecipitation with FLAG-α2 chimaerin there was no detectable pull down of CRMP-2 in the absence of PMA (Fig. 5.4 lane2). However in the presence of PMA, full length α2 chimaerin coprecipitated CRMP-2 (Fig. 5.4 lane 6) and the presence of the N-terminus (1-196) of α2 chimaerin, containing the putative SH2 domain was required for association with CRMP-2.

5.B.3 α2 chimaerin potentially interacts with multiple regions of CRMP-2

To determine the region of CRMP-2 responsible for α2 chimaerin interaction, Cos-7 cells were transiently transfected with HA-α2 chimaerin and GST fused CRMP-2 constructs and treated with PMA before pulling out GST constructs with Glutathione agarose.

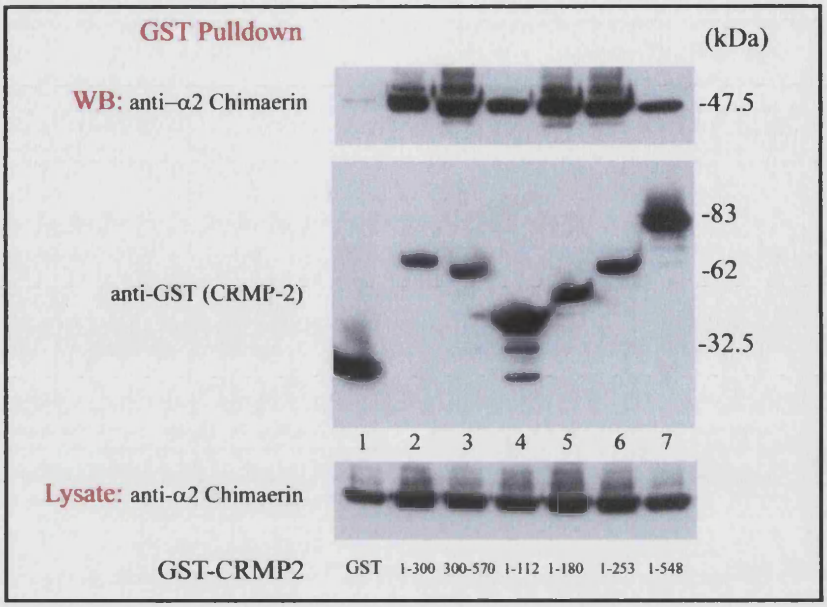


Fig 5.5 GST-CRMP-2 pulldowns from transfected Cos-7 cells.

Cos-7 cells were transiently transfected with HA-α2 Chimaerin and various GST-CRMP-2 constructs. Cells were treated with 1 μM PMA for 1 hr before carrying out GST pulldowns. Samples were analysed by SDS gel electrophoresis and western blotted with indicated antibodies.

In the presence of PMA, α2 chimaerin can associate with both N- and C-terminal halves of CRMP-2 (Fig. 5.5 lane 2 and 3) and various N-terminal fragments, indicating possible multiple regions of association. To examine this further, immunoprecipitations from cells expressing these and other C-terminal CRMP-2 constructs and the isolated SH2 domain of α2 chimaerin should be carried out in the absence of PMA.

5.B.4 CRMP-2 association with α2 chimaerin is independent of phosphotyrosine, in the presence of PMA

Having identified the N-terminal of α2 chimaerin, encompassing the SH2 domain, as responsible for its *in vivo* interaction with CRMP-2 it was of interest to determine if this interaction could be phosphotyrosine regulated. To accomplish this, α2 chimaerin mutants possessing SH2 mutations R56L and R73L which have been shown to

eliminate phosphotyrosine binding *in vitro* (Hall et al., 2001) were used in immunoprecipitation studies of FLAG-CRMP-2, in Cos-7 cells in the presence of PMA.

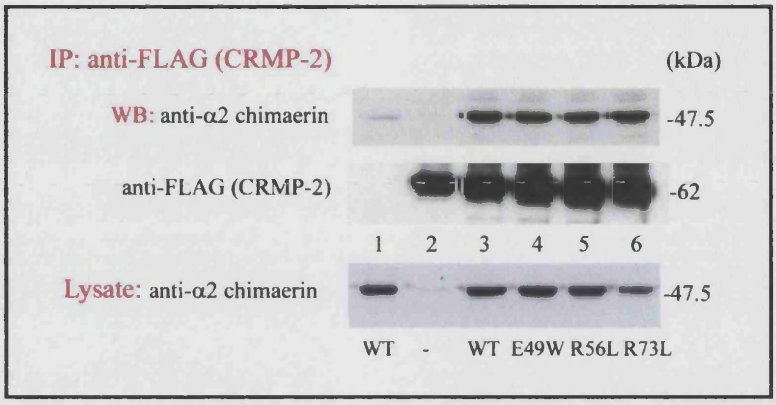


Fig. 5.6 FLAG-CRMP-2 co-immunoprecipitation of SH2 mutated α2 chimaerin.
Cos-7 cells were transfected with HA-α2 chimaerin constructs (lanes 1 and 3-6) and FLAG-CRMP-2 (lanes 2-6) and treated with 10 μM PMA for 1hr before lysis and immunoprecipitation with anti-FLAG antibody. Samples were analysed by SDS gel electrophoresis and western blotted with indicated antibodies.

Co-expression of CRMP-2 and α2 chimaerin in Cos-7 cells, followed by PMA treatment promotes the association of these two proteins. In the presence of PMA the association of α2 chimaerin and CRMP-2 does not require tyrosine phosphorylation as α2 chimaerin mutated proteins, R56L and R73L, which are incapable of phosphotyrosine interactions *in vitro*, were still able to associate with CRMP-2 (Fig. 5.6 lane 5 and 6).

5.B.5 Pervanadate treatment enhances α2 chimaerin association with CRMP-2 in N1E-115 neuroblastoma cells

The previous result indicated CRMP-2 and α2 chimaerin can associate, with impaired SH2 function, in the presence of PMA. To further investigate whether tyrosine phosphorylation plays a part the association of these two molecules, immunoprecipitations from Cos-7 cells over expressing FLAG-CRMP2 and HA-α2

chimaerin were carried out, following treatment with the tyrosine phosphatase inhibitor pervanadate (peroxides of vanadate; Kadota et al., 1987).

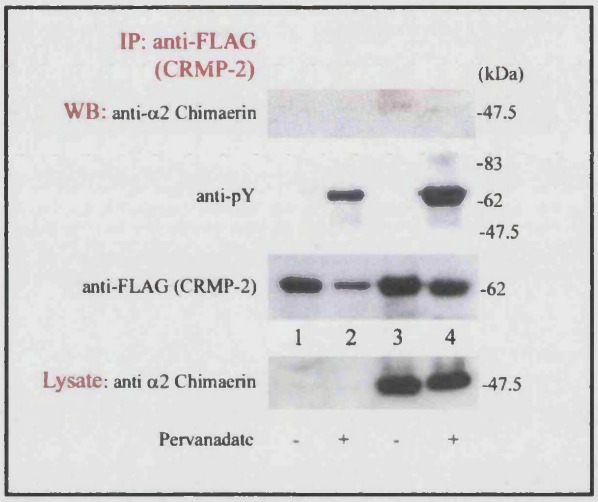


Fig. 5.7 Pervanadate treatment does not effect co-precipitation of α2 chimaerin with FLAG-CRMP-2 in Cos-7 cells. Cos-7 cells were transfected with FLAG-CRMP-2 alone (lanes 1+2) or co-transfected with HA-α2 Chimaerin (lane 3 and 4) and after 16hr expression were treated with 100 μM pervanadate (lane 2 and 4) or H_2O_2 control (lane 1 and 3) for 20min before harvesting, lysis and immunoprecipitation with anti-FLAG antibody. Samples were analysed by SDS gel electrophoresis and western blotted with indicated antibodies.

Pervanadate treatment of Cos-7 cells substantially increased tyrosine phosphorylation of CRMP-2 (Fig. 5.7 lane 2 and 4) and α2 chimaerin (data not shown). Thus in Cos-7 cells endogenous tyrosine kinases are capable of phosphorylating both CRMP-2 and α2 chimaerin. Pervanadate treatment however had no effect on the association of CRMP-2 and α2 chimaerin which remained negligible (lane 3 and 4). This finding is consistent with the previous result where mutations of the SH2 domain demonstrated interactions between these two proteins are independent of tyrosine phosphorylation (Fig. 5.6). However the consequence of tyrosine phosphorylation of α2 chimaerin remains unclear. To further investigate whether tyrosine phosphorylation of CRMP-2 can promote α2 chimaerin interaction in a neuronal environment, these studies were repeated in N1E-115 neuroblastoma cells.

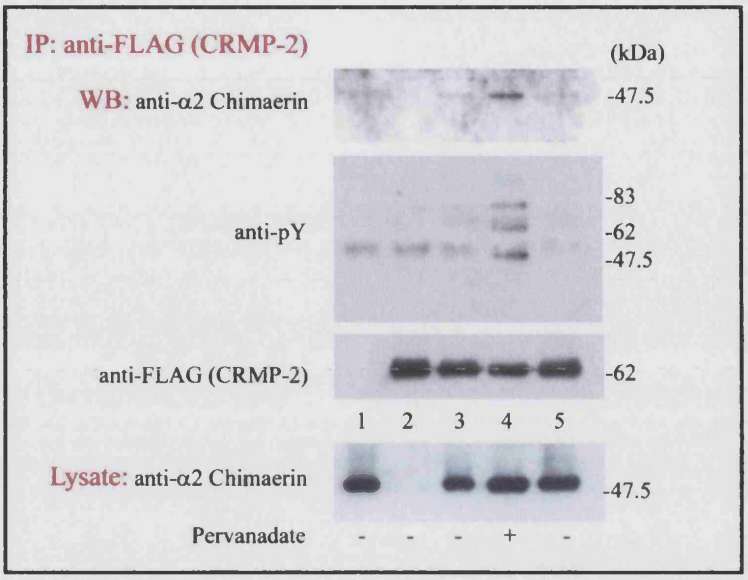


Fig. 5.8 Pervanadate treatment enhances co-precipitation of α2 Chimaerin with FLAG-CRMP-2 in neuroblastoma cells. N1E-115 neuroblastoma cells were transfected with HA-α2 chimaerin (Lanes1,3, 4 + 5) and FLAG-CRMP-2 (lanes 2-5) and after 16 hrs expression were treated with 100 μM pervanadate (lane 4) or Catalase/H_2O_2 control (lane 5) for 20 min before lysis and immunoprecipitation with anti-FLAG antibody. Samples were analysed by SDS gel electrophoresis and western blotted with indicated antibodies.

N1E-115 neuroblastoma cells in serum were transiently transfected with CRMP-2 and α2 chimaerin. Co-immunoprecipitation of α2 chimaerin with FLAG-CRMP-2 was negligible under unstimulated conditions (Fig. 5.7 lane3 and 5). Treating N1E-115 neuroblastoma cells with 100μM pervanadate increased the association of over-expressed CRMP-2 and α2 chimaerin (Fig.5.7 lane4). The phosphotyrosine blot of the immunoprecipitate, showed potential tyrosine phosphorylation of CRMP-2 is slightly increased upon pervanadate treatment and there are at least two associated tyrosine phosphorylated proteins. Thus in neuroblastoma cells, CRMP-2 exists in a dynamic state of tyrosine phosphorylation/dephosphorylation and treatment with pervanadate shifts the equilibrium to the phosphorylated state and at the same time enhances α2 chimaerin association.

It is possible the associated tyrosine phosphorylated protein which co-migrates with α2 chimaerin (47kDa) is α2 chimaerin in a phosphorylated state but the ~80kDa protein remains elusive. This could potentially represent one of the recently identified CRMP isoforms, as CRMPs have been shown to exist as heterotetramers (Wang et al., 1997, Leung et al., 2002) and splice variant isoforms of all CRMP members (1-5) have been found of ~80kDa (Leung et al., 2002, Yuasa-Kawada et al., 2003).

The increase in association of CRMP-2 with α2 chimaerin could be explained by classical SH2/phosphotyrosine association. However α2 chimaerin appears itself tyrosine phosphorylated and the consequences of this is unknown. Alternatively tyrosine kinase signalling events, upregulated by pervanadate treatment, may increase CRMP-2 and α2 chimaerin association independent of direct tyrosine phosphorylation e.g by recruitment and activation of PLCγ, leading to DAG production and subsequent chimaerin activation. It was of interest to note N1E-115 neuroblastoma cells collapsed upon pervanadate treatment and therefore potential neurite collapsing signals may promote CRMP-2/α2 chimaerin interaction. An alternative possibility would be tyrosine phosphorylation signalling induces the interaction of CRMP-2 with α2 chimaerin, which subsequently promotes collapse.

The different result between cell lines are indicative of neuronal specificity, potentially a neuronal specific tyrosine kinase is required to phosphorylate CRMP-2 to increase α2 chimaerin association. No co-precipitation of any tyrosine phosphorylated proteins was observed with immunorecipitated CRMP-2 from Cos-7 cells (Fig 5.7), highlighting the difference in signalling and binding partners between the two cell types. Pervanadate treatment of N1E-115 neuroblastoma cells causes neurite retraction, while in Cos-7 cells it lead to dramatic cell ruffling and endocytosis (data not shown). These cell specific responses are also generated by PMA treatment and could possibly,

in part, be attributed to the presence of endogenous levels of α2 chimaerins in the neuroblastoma cells. Further immunoprecipitations following expression of the isolated SH2 domain of α2 chimaerin and CRMP-2 constructs in pervanadate treated neuroblastoma cells could possibly map any region of phosphotyrosine regulated interaction.

In an attempt to determine whether this was a direct pY CRMP-2 and α2 chimaerin SH2 interaction, immunoprecipitationss were carried out from N1E-115 neuroblastoma cells expressing CRMP-2 and the mutant α2 chimaerin R73L, with eliminated phosphotyrosine binding ability, after treatment with pervanadate.

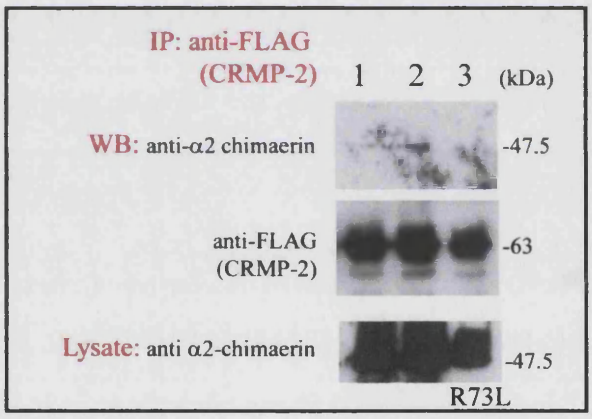


Fig. 5.9 α2 Chimaerin R73L was not co-precipitated with FLAG-CRMP-2 in pervanadate treated neuroblastoma cells. N1E-115 neuroblastoma cells were transfected with HA-α2 chimaerin and FLAG-CRMP-2 and after 16 hrs expression were treated with 100 μM pervanadate for 20 min before lysis and immunoprecipitation with anti-FLAG antibody. Samples were analysed by SDS gel electrophoresis and western blotted with indicated antibodies.

Treating neuroblastoma cells with pervanadate enhanced the association of over-expressed CRMP-2 and α2 chimaerin (Fig. 5.8 and 5.9 lane 1 and 2). Mutant α2 chimaerin R73L did not bind CRMP-2 in pervanadate treated neuroblastoma cells suggesting tyrosine phosphorylation binding of α2 chimaerin may be responsible for this increased association, presumably directly to CRMP-2.

5.C Tyrosine Phosphorylation of α2 Chimaerin

5.C.1 α2 chimaerin is tyrosine phosphorylated and associates with tyrosine phosphorylated proteins *in vivo*

From the previous experiment involving pervanadate treatment, it appears α2 chimaerin is itself a target of endogenous tyrosine kinases in both Cos-7 and N1E-115 neuroblastoma cells (data not shown and Fig.5.8). To substantiate this and to investigate phosphotyrosine associations of α2 chimaerin, a cell line permanently expressing α2 chimaerin (α2.13) was treated with combinations of PMA and pervanadate.

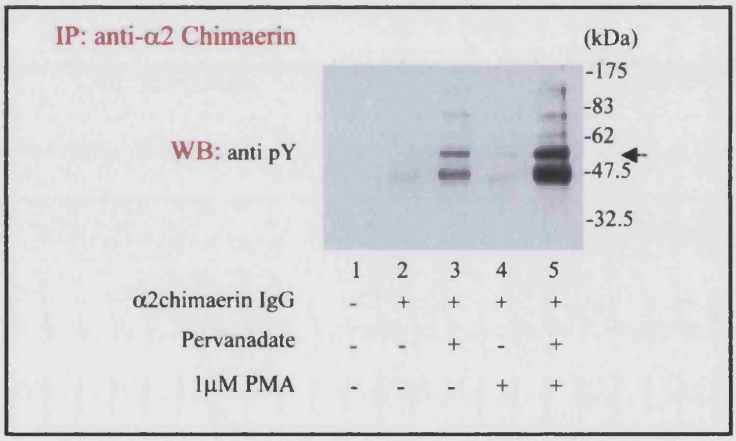


Fig. 5.10 α2 chimaerin is tyrosine phosphorylated *in vivo*.
α2.13 neuroblastoma cell lines were treated with +/- pervanadate for 20 min +/- 1 μM PMA for 1hr before lysis and immunoprecipitation with anti-α2 chimaerin antibody. Western blots were then probed with anti-phosphotyrosine antibody.

Immunoprecipitation of α2 chimaerin from α2.13 neuroblastoma cell lines resulted in no detectable association of any tyrosine phosphorylated proteins and minimal tyrosine phosphorylation of α2 chimaerin itself (Fig. 5.9 lane 2). Treatment with pervanadate increased tyrosine phosphorylation of a band corresponding to α2 chimaerin and allowed the detection of two associated tyrosine phosphorylated proteins (~55kDa and ~80kDa) (Fig. 5.9 lane3). PMA treatment alone also resulted in the slight detection of a

~55kDa tyrosine phosphorylated protein but no increase in tyrosine phosphorylation of α2 chimaerin (Fig. 5.9 lane 4). A combination of PMA plus pervanadate led to a considerable increase in tyrosine phosphorylation of α2 chimaerin and an increase in association/phosphorylation of the ~55kDa protein (Fig. 5.9 lane 5 arrow). No significant increase association/phosphorylation of the 80kDa protein, compared with pervanadate treatment alone, was apparent, but minor ~62 kDa and ~100kDa bands were also observed under these conditions. The 62kDa band was not CRMP-2 as determined by western blotting (data not shown).

These increases in phosphotyrosine bands could represent an increase in association of a phosphotyrosine containing protein (potentially via the SH2 domain) or alternatively an increase in phosphorylation of an associated protein. Tyrosine phosphorylation of α2 chimaerin itself could also recruit other SH2/PTB containing proteins, which may themselves be substrates for tyrosine kinases. It is also highly possible none of these associated proteins are directly bound to α2 chimaerin but are members of a complex.

The increase in tyrosine phosphorylation of a band corresponding to α2 chimaerin with pervanadate, and its further increase with PMA plus pervandate, indicates the potential association of α2 chimaerin with a tyrosine kinase which is promoted by PMA, or the activation of tyrosine kinases by PMA treatment. The lack of α2 chimaerin tyrosine phosphorylation upon PMA treatment alone would however therefore argue for rapid phosphatase activity associated with α2 chimaerin. The ~80kDa protein associated with α2 chimaerin upon pervanadate treatment is unaffected by further phorbol ester treatment. This result could imply not all protein interactions of α2 chimaerin are increased by phorbol ester treatment although whether this represents a direct association is unclear. It was of interest to determine if the 55kDa protein was β

tubulin (55kDa), which has previously been found to associate with α2 chimaerin SH2 domain *in vitro* (Ferrari Thesis 1999). Western blotting did not detect any associated β tubulin (data not shown). It is tempting to speculate the 55kDa protein represents a tyrosine kinase/phosphatase.

Tyrosine phosphorylation of α2 chimaerin and associated proteins was also investigated in transiently transfected N1E-115 neuroblastoma cells expressing both full length and various FLAG-N-terminal deleted fragments of α2 chimaerin. These were treated with pervanadate and immunoprecipitated with FLAG antibody to determine regions of α2chimaerin involved in binding.

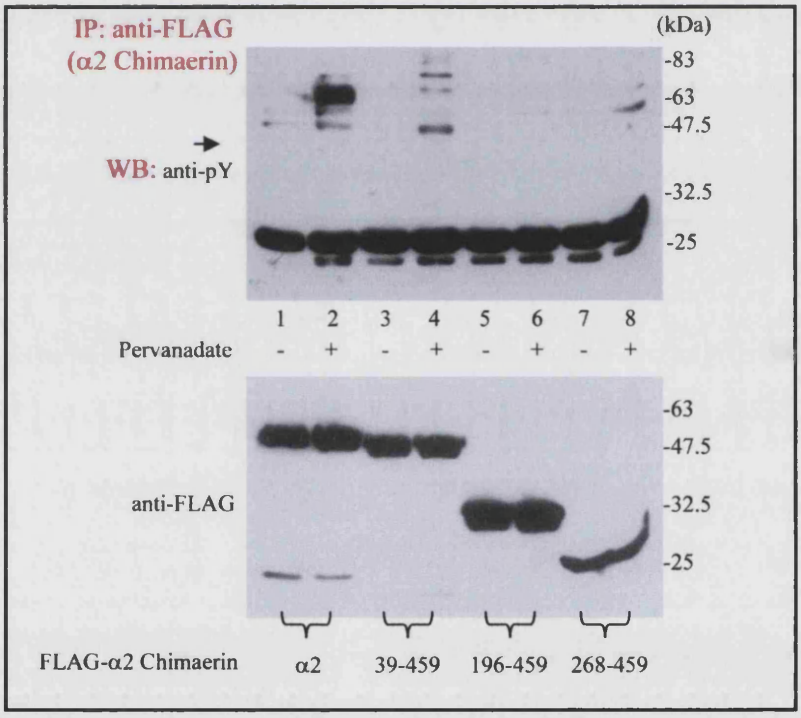


Fig. 5.11 Associated tyrosine phosphorylated proteins of α2 chimaerin from neuroblastoma cell immunoprecipitations.

N1E-115 neuroblastoma cells were transiently transfected with FLAG α2 chimaerin constructs and treated with +/- pervanadate before immunoprecipitation with anti-FLAG antibody. Samples were analysed by SDS gel electrophoresis and western blotted with indicated antibodies.

Immunoprecipitation of full length FLAG-α2 chimaerin from transiently transfected N1E-115 neuroblastoma cells, and probing with a phosphotyrosine antibody showed the presence of a weakly tyrosine phosphorylated band corresponding in mobility to α2

chimaerin (Fig. 5.10 lane 1 arrow). Treatment with pervandate prior to FLAG immunoprecipitation of full length FLAG-α2 chimaerin only slightly increased tyrosine phosphorylation of this band (α2 chimaerin) but significantly increased the detection of associated tyrosine phosphorylated proteins, namely a ~ 65 kDa protein (Fig. 5.10 lane 2). Interestingly the population of α2 chimaerin associated phosphotyrosine proteins recovered from transiently transfected cells differed from those of permanent α2 chimaerin cell line, α2.13 (Fig.5.9).

Deletion of the N-terminal 38 residues of α2 chimaerin also had profound effects on α2 chimaerin phosphotyrosine associations and enhanced tyrosine phosphorylation of α2 chimaerin upon pervanadate treatment (Fig. 5.10 lane 4). This result could implicate these N-terminal residues in a native inhibited conformation of α2 chimaerin. The ~65 kDa major phosphoprotein, which bound to full length α2 chimaerin, was also lost with removal of N-terminal 38 residues, implicating these residues in protein associations. Thus the ~65 kDa protein could perhaps represent an anchoring protein which associates with inhibited conformation of α2 chimaerin.

Removal of the N-terminal 195 residues, including the SH2 domain, abolished all tyrosine phosphorylation of α2 chimaerin, upon pervanadate treatment, and also resulted in the loss of the majority of associated tyrosine phosphorylated proteins. Thus α2 chimaerin N-terminal 195 residues most likely encompasses the target sites for tyrosine kinases as well as residues (SH2 domain) for the interaction with several tyrosine phosphorylated proteins. However these differences may also be a result of altered conformation of these fragment proteins. Due to background bands it was also impossible to determine the phosphorylation state of the isolated GAP domain (268-459 Fig. 5.10 lane8), although increased detection of a ~55 kDa phosphotyrosine protein was observed upon pervanadate treatment, which may represent a specific cytosolic

GAP domain associating phosphoprotein. Again these associations may well be indirect.

5.C.2 Fyn phosphorylates α2 chimaerin *in vitro*

Fyn non receptor tyrosine kinases are members of the Src family and one isoform (59 kDa) is highly enriched in the brain where it is neurone specific and localised to axonal tracts and growth cones (Bare et al., 1993, Bixby et al., 1993). NCAM dependent neurite outgrowth is inhibited in Fyn -/- mice (Beggs et al., 1994) and it is believed NCAM, FAK and Fyn form a functional adhesion signalling complex modulating growth cone motility (Beggs et al., 1997). Fyn has been shown to interact with and phosphorylate Tau (Lee et al., 1998) and was recently implicated in Sema 3A induced growth cone collapse where it tyrosine phosphorylates (Y15) and activates Cdk5 (Sasaki et al., 2002). p190RhoGAP and p250RhoGAP are also target of Fyn during oligodendrocyte differentiation (Wolf et al., 2001, Taniguchi et al.,). It is therefore possible that Fyn kinase might be responsible for tyrosine phosphorylation of α2 chimaerin. To test this *in vitro* kinase assays with purified Fyn (Upstate) and FLAG immunoprecipitated α2 chimaerin proteins from Cos-7 cells were undertaken.

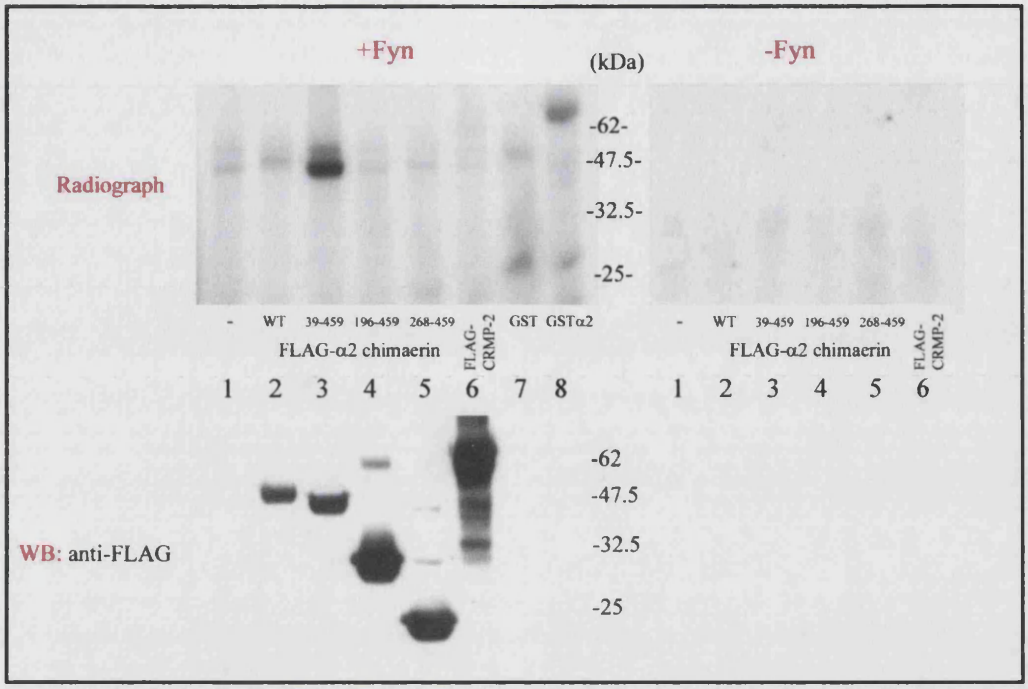


Fig. 5.12 Fyn *in vitro* kinase assay with immunoprecipitated α2 chimaerin proteins.

Cos 7 cells were transiently transfected with FLAG constructs and allowed to express for 16hr before immunoprecipitations with anti-FLAG antibody were carried out. Isolated substrate proteins were re-suspended in kinase buffer (5μCi ^{32}PγATP, 100 μM ATP, 25 mM Tris-HCl pH7.2, 31.25 mM $MgCl_2$, 6.25 mM $MgCl_2$M 0.5 mM EGTA). *In vitro* kinase assays were then undertaken on these samples and recombinant GST proteins (5μg), using purified Fyn (2 units) and incubated at 30 ^{0}C for 30 min. Samples were analysed by SDS gel electrophoresis, and exposed to film before western blotting with indicated antibodies.

Immunoprecipitation of FLAG-α2 chimaerin proteins, from Cos-7 cells followed by *in vitro* kinase assays demonstrated Fyn was not capable of phosphorylating full length α2 chimaerin (Fig. 5.11 lane 2). However deletion of the N-terminal 38 residues of α2 chimaerin resulted in clear phosphorylation by Fyn (Fig. 5.11 lane 3). This implies that the N-terminal 38 residues masks Fyn kinase sites and is consistent with a closed conformation of α2 chimaerin. It was of interest to note there was minimal phosphorylation of bacterial recombinant GST α2 chimaerin (Fig. 5.11 lane 8). Further deletion of the N-terminal 195 residues completely abolished phosphorylation by Fyn (Fig. 5.11 lane 4) indicating that phosphorylation target sites are likely to reside within

residues 39-196, although further conformational hindrances are possible. This region contains the SH2 domain and several tyrosine residues which are candidates for phosphorylation (Fig. 5.12). Although no phosphorylation by endogenous associated Cos-7 cell kinases occurred, it is possible the addition of purified Fyn protein could activate any associated kinases with misleading results. However some direct phosphorylation of recombinant GST-α2 chimaerin was demonstrated (last lane).

α2 chimaerin (1-240)

```
1   maltlfdtde yrppvwksyl yqlqqeaphp rritctceve nrpkyygref hgmisreaad
61  qllivaegsy liresqrqpg tytlalrfgs qtrnfrlyyd gkhfvgekrf esihdlvtdg
121 litlyietka aeyiakmtin piyehvgytt lnrepaykkh mpvlkethde rdstgqdgvs
181 ekrltslvrr atlkeneqip kyekihnfkv htfrgphwce ycanfmwgli aqgvkcadcg
```

XXX= 39-196

RED = SH2 Domain

y = potential tyrosine phosphorylation site

Fig. 5.13 α2 chimaerin N-terminal (1-240) peptide sequence

5.D CRMP-2 Phosphorylation

5.D.1 CRMP-2 tyrosine phosphorylation

When expressed in either neuroblastoma cells or Cos-7 cells treated with pervanadate, tyrosine phosphorylation of CRMP-2 was observed (Fig. 5.7 and 5.8). CRMP-2 is a highly expressed neuronal phosphoprotein which has been implicated in Sema 3A collapse (Goshima et al., 1995), a pathway involving Fyn activity (Sasaki et al., 2002). CRMP-2 has been shown tyrosine phosphorylated during Sema 3A induced collapse by Fps/Fes tyrosine kinase (Mitsui et al., 2002). It was of interest to determine whether Fyn could phosphorylate CRMP-2. From the *in vitro* kinase assay it appears Fyn is not capable of phosphorylating full length CRMP-2 synthesised in Cos-7 cells (Fig 5.12 lane 6), although this does not preclude *in vivo* phosphorylation.

5.D.2 CRMP-2 is phosphorylated on Ser^{522} *in vitro* by Cdk5 (p25/Cdk5)

CRMP-2 is highly Ser/Thr phosphorylated *in vivo* (Gu et al., 2000) and ROK has been identified as a kinase responsible, phosphorylating Thr^{555} during LPA, but not Sema 3A induced growth cone collapse (Aimura et al., 2000), although CRMP-2 has also been implicated in Sema 3A collapse. Cdk5 activity has also recently been implicated in Sema 3A collapse (Sasaki et al., 2002) and since both these proteins have been identified as α2 chimaerin interactors the possibility that CRMP-2 could serve as a substrate for Cdk5 activity was investigated.

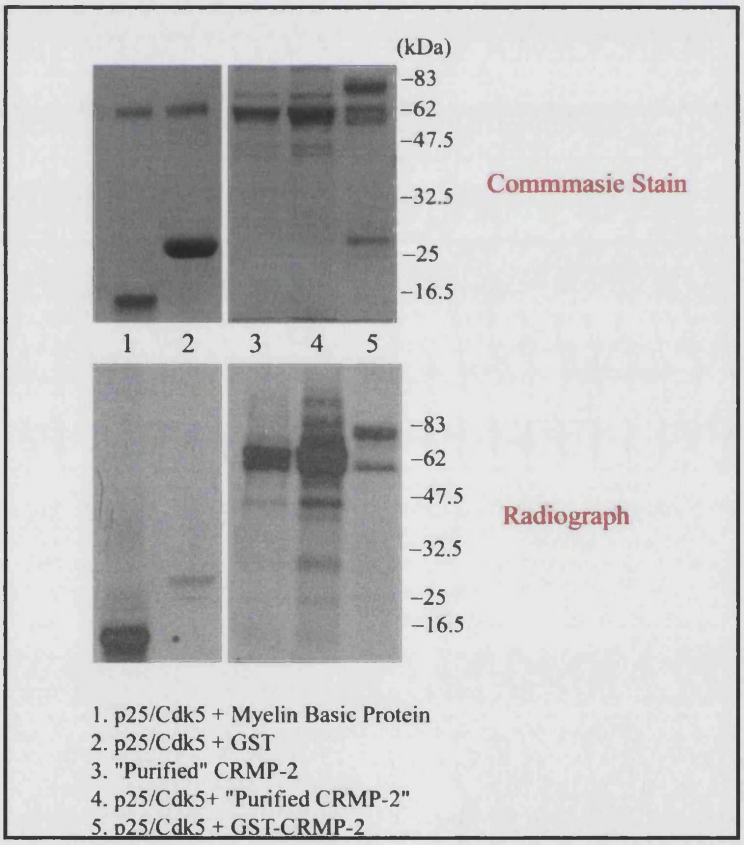


Fig. 5.14 p25/Cdk5 *in vitro* kinase assay with CRMP-2.

5 μg of recombinant protein or purified rat brain protein was mixed with 2 μl recombinant p25/Cdk5 in a 30μl Reaction (5 μCi $^{32}P\gamma ATP$, 100 μM ATP, 20 mM MOPS pH 7.4, 5 mM $MgCl_2$) and incubated at 30 ^0C for 30 min. Samples were analysed by SDS gel electrophoresis and exposed to film.

In vitro kinase assay revealed the "purified" CRMP-2 from rat brain extract had associated kinase activity (Fig. 5.14 lane 3) but phosphorylation was still enhanced upon the addition of recombinant p25/Cdk5 (Fig. 5.14 lane 4). p25/Cdk5 was also capable of phosphorylating recombinant GST-CRMP-2 (Fig. 5.14 lane5) thus identifying it as a potential novel substrate for Cdk5 activity. Kinase assays were however extremely variable with different recombinant GST-CRMP-2 preparations. This could be because post translational modifications serve to regulate Cdk5 phosphorylation of CRMP-2, such as phosphorylation by other kinases, which is the case for Tau phosphorylation (Sengupta et al., 1997)

CRMP-2 contains seven minimal Cdk5 consensus sites (S/TP) with S^{522}PAK matching the preferred consensus sequence S/TPXK/R (Fig. 5.15). CRMP-2 is phosphorylated on Thr^{509}, Ser^{518} and Ser^{522} in association with Neurofibrillary tangles (NFT) in tissue from Alzheimer disease patients (Gu et al., 2000); where p25/Cdk5 accumulation has also been noted (Patrick et al., 1999). It was therefore sought to determine whether any of these *in vivo* sites were Cdk5 phosphorylation sites, by using site directed mutagenesis to change Ser/Thr to non phosphorylatable residues.

```
  1 msyqgkknip ritsdrllik ggkivnddqs fyadiymedg likqigenli vpggvktiea
 61 hsrmvipggi dvhtrfqmpd qgmtsaddff qgtkaalagg ttmiidhvvp epgtsllaaf
121 dqwrewadsk sccdyslhvd itewhkgiqe emealvkdhg vnsflvymaf kdrfqltdsq
181 iyevlsvird igaiaqvhae ngdiiaeeqq rildlgitgp eghvlsrpee veaeavnrsi
241 tianqtncpl yvtkvmsksa aeviaqarkk gtvvygepit aslgtdgshy wsknwakaaa
301 fvtspplspd pttpdflnsl lscgdlqvtg sahctfntaq kavgkdnftl ipegtngtee
361 rmsviwdkav vtgkmdenqf vavtstnaak vfnlyprkgr isvgsdadlv iwdpdsvkti
421 sakthnsale ynifegmecr gsplvvisqg kivledgtlh vtegsgryip rkpfpdfvyk
481 rikarsrlae lrgvprglyd gpvcevsvtp ktvtpassak tspakqqapp vrnlhqsgfs
 541 lsgaqiddni prrttqriva ppggranits lg

s/tp = minimal cdk5 consensus
s/t  = phosphorylated in Alzheimer's Disease (509,518,522)
```

Fig. 5.15 CRMP-2 Peptide Sequence with Highlighted Phosphorylation Sites

To map the potential sites of phosphorylation, site directed mutagenesis of FLAG-CRMP-2 was undertaken to produce the mutants T509A and S522A (see methods). These constructs were expressed in Cos-7 cells and the FLAG immmunoprecipitated proteins were used in an *in vitro* kinase assay with recombinant p25/Cdk5 (Fig. 5.16).

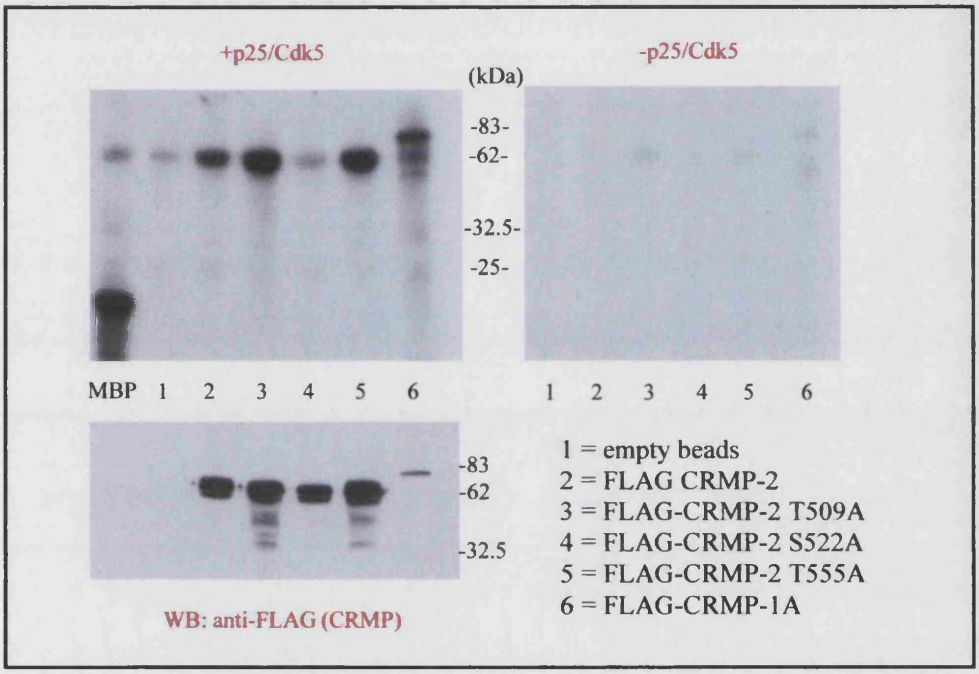


Fig. 5.16 p25/Cdk5 *in vitro* kinase assay with immunoprecipitated CRMP-2 proteins.
Cos-7 cells were transiently transfected with FLAG constructs and allowed to express for 16hr before immunoprecipitations with anti-FLAG antibody were carried out to isolate the substrate proteins which were re-suspended in kinase buffer (5μCi ^{32}PγATP, 100 μM ATP, 20 mM MOPS pH7.4, 5 mM $MgCl_2$,). *In vitro* kinase assays were undertaken on these samples using recombinant p25/Cdk5 and incubated at 30 ^{0}C for 30 min. Samples were analysed by SDS gel electrophoresis, and exposed to film before western blotting with indicated antibodies.

From these results, CRMP-2 Ser^{522} appears the major site for p25/Cdk5 phosphorylation of CRMP-2 as introducing the mutation S522A eliminated all observed phosphorylation by p25/Cdk5(Fig. 5.16 lane 4). This site is conserved in other CRMP family members as well as across species (CRMP-1 & CRMP-4 Fig 5.16) and p25/Cdk5 was found to strongly phosphorylate CRMP-1A (p80) (Fig. 5.16 lane 6).

```
CRMP-1 (rat)      VYEVPATPKH--AAPAPSAKSSPSKHQPPP
CRMP-2 (rat)   503VCEVSVTPKT--VTPASSAKTSPAKQQAPP
CRMP-3 (rat)      VHEVMLPAKP--GSGTQARASCPGKISVPP
CRMP-4 (mouse)    VFDLTTTPKG--GTPAGSARGSPTRPNPP-
CRMP-5 (rat)      VAIVVHPGKKEMGTPLADTPTRPVTRHGG-
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Fig. 5.17 Alignment of CRMP family members

5.D.3 CRMP-2 associates with p35/Cdk5N144 *in vivo*

To investigate the possible associations of CRMP-2 with p35/Cdk5, immunoprecipitations from Cos-7 cells expressing combinations of proteins were carried out.

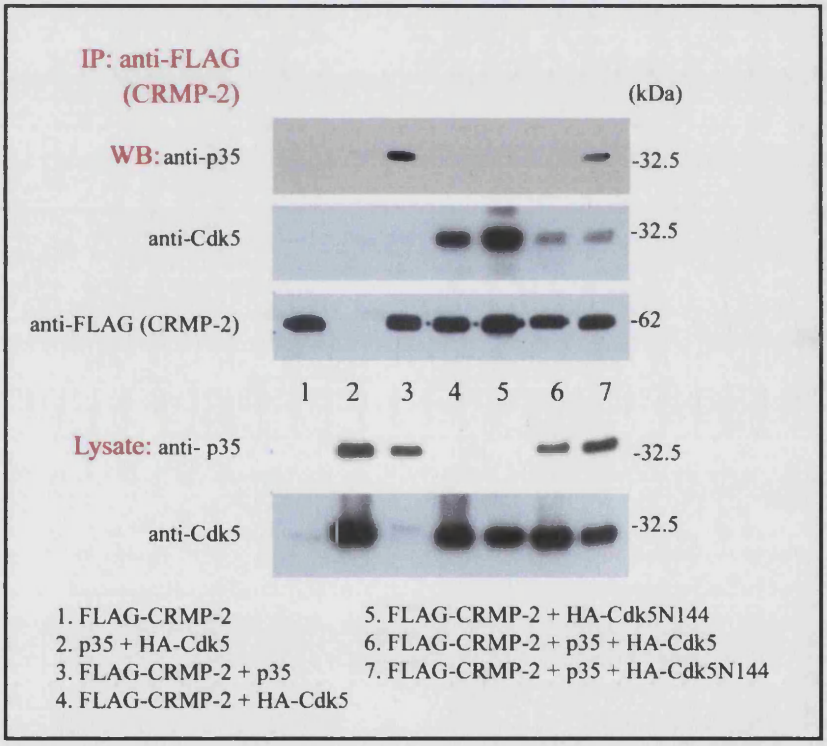

Fig. 5.18 FLAG-CRMP-2 immunoprecipitations from transfected Cos-7 cells.
Cos-7 were transiently transfected with combinations of FLAG-CRMP-2, p35 and HA-Cdk5. 16hrs post transfection cells were harvested and immunoprecipitated with anti- FLAG antibody. Samples were analysed by SDS gel electrophoresis and western blotted with indicated antibodies.

P35 and Cdk5 were both found to co-precipitate with FLAG-CRMP-2 immunoprecipitated from Cos-7 cells co-expressing the relevant proteins (Fig. 5.18 lane 3 and 4). A potential trimeric complex of p35/Cdk5N144 and CRMP-2 was also pulled out with use of kinase-dead Cdk5N144 (Fig. 5.18 lane 7) but not with active kinase (Fig. 5.18 lane 6). Cdk5N144 forms a more stable complex with p35 than active Cdk5 and this may account this difference. This result is consistent with a substrate role of CRMP-2 for p35/Cdk5 kinase activity.

5.D.4 GSK-3 β phosphorylates CRMP-2 *in vitro*

The ability of GSK3β to phosphorylate CRMP-2 was investigated. GSK3β is another proline directed Ser/Thr kinase, but which mainly phosphorylates primed substrates. Priming involves previous phosphorylation by another kinase, 4 residues C-terminus of the target site (S/TXXXpS/T) (Harwood et al., 2001). Cdk5 has been shown to potentiate GSK3β, phosphorylation of Tau, with both kinases responsible for most of the abnormal phosphorylation of Tau observed in tissue extract from Alzheimers disease patients. CRMP-2 has also been found hyperphosphorylated associated with NFT in tissue from Alzheimers Disease patients. The possibility that p35/Cdk5 could cooperate with GSK-3β to bring about increased phosphorylation of CRMP-2 could also have implications in Sema3A induced growth cone collapse where CRMP-2 (Goshima., et al 1995), Cdk5 (Sasaki., et al 2002) and GSK3β (Eickholt., et al 2002) activity have all been implicated.

All seven S/TP sites in CRMP-2 (Fig. 5.15) are potential targets of GSK3β. In addition phosphorylation of Ser^{522} could also self prime for Ser^{518} phosphorylation ($S^{518}AKTS^{522}PAK$). To investigate this, several more point mutations of CRMP-2 were

made. FLAG CRMP-2 mutants were expressed in Cos-7 cells and immunoprecipitated. These proteins were used in an *in vitro* kinase assay with recombinant GSK3β (Upstate)

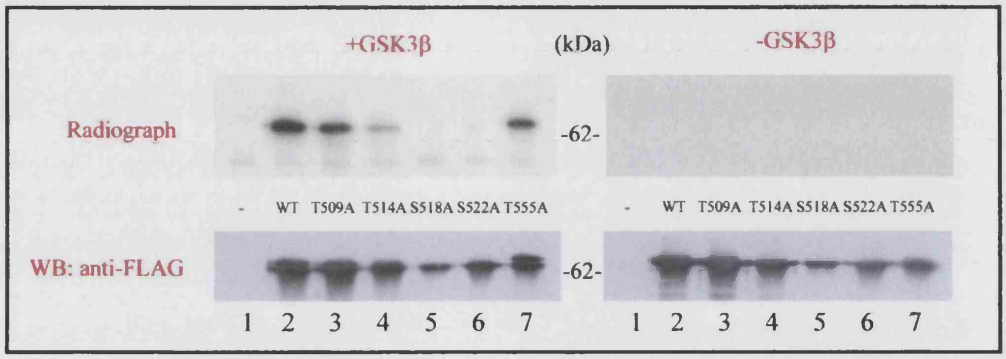

Fig. 5.19 GSK3β *in vitro* kinase assay with CRMP-2 proteins

Cos 7 cells were transiently transfected with FLAG constructs and allowed to express for 16hr before immunoprecipitations with anti-FLAG antibody were carried out to isolate the substrate proteins which were re-suspended in kinase buffer (5μCi ^{32}PγATP, 100μM ATP, 2mM MOPS pH7.4, 50μM EDTA, 2.5mM magnesium actetate). *In vitro* kinase assays were undertaken on these samples using recombinant GSK3β (50ng) and incubated at 30^{0}C for 30min. Samples were analysed by SDS gel electrophoresis, and exposed to film before western blotting with indicated antibodies.

From the results of the *in vitro* kinase assay, CRMP-2 appears to be a novel substrate for GSK3β activity (Fig. 5.19 lane 2). Mutation of CRMP-2 S522A and S518A completely eliminated phosphorylation by GSK3β (lane 5 and 6) while T514A diminishes phosphorylation levels considerably but not totally (lane 3), implying there are possible multiple sites. One possibility is that mutations in this general area of CRMP-2 disrupt GSK phosphorylation making it impossible to define the sites by this mutational approach. However an alternative model is possible. The total loss of phosphorylation by GSK3β of CRMP-2 S522A potentially implicates this as an initiating phosphorylation site. However since CRMP-2 S518A mutant also completely knocks out all GSK3β phosphorylation, this may imply that Ser^{522} is not actually a site

for GSK3β activity, but is in fact an essential phosphorylation/priming site for subsequent GSK3β mediated phosphorylation.

Previous work had ruled out the possibility of GSK3β phosphorylating CRMP-2 *in vitro* (Gu et al., 2000). These authors used recombinant CRMP-2 protein as a substrate which would yield negative results if previous priming was required, as suggested by results described here (Fig. 5.19). Cos-7 lysates have been shown to posses endogenous kinases capable of phosphorylating CRMP-2 (Gu et al., 2000). Thus having immunoprecipitated FLAG-CRMP-2 constructs from Cos-7 it is conceivable that they were phosphorylated on Ser^{522} within the cell, priming for subsequent GSK3β phosphorylation of Ser^{518} followed by Thr^{514}. The use of the mutant GSK3β R96A which has a reduced ability to phosphorylate primed but not unprimed sites (Frame et al., 2001) could clarify this.

p25/Cdk5 kinase assays were repeated with CRMP-2 T514A and S518A to confirm they were still capable substrates for Cdk5 activity (Fig. 5.10).

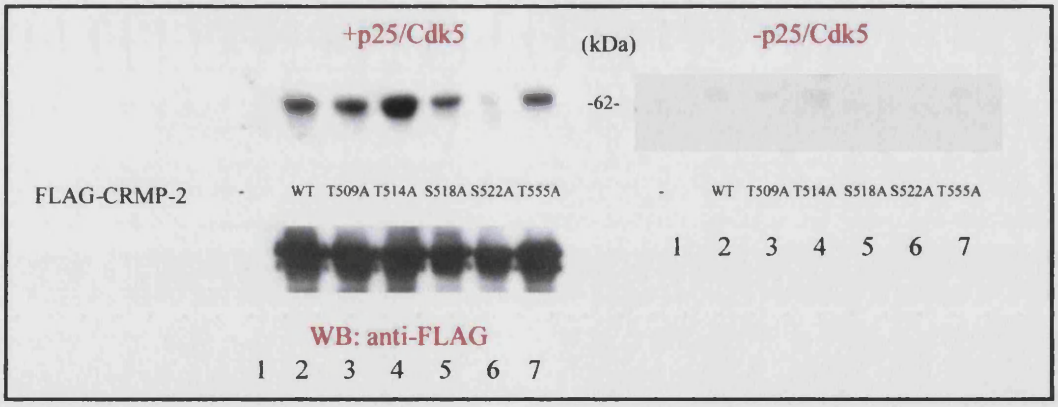

Fig.5.20 p25/Cdk5 *in vitro* Kinase Assay with Mutated CRMP-2 Proteins.
Cos 7 cells were transiently transfected with FLAG constructs and allowed to express for 16hr before immunoprecipitations with anti-FLAG agarose conjugated antibody were carried out. The isolated substrate proteins were re-suspended in kinase buffer (5 μCi $^{32}P\gamma ATP$, 100 μM ATP, 20 mM MOPS pH7.4, 5 mM $MgCl_2$,) and *in vitro* kinase assays were undertaken, using recombinant p25/Cdk5, incubating at 30 ^0C for 30min. Samples were analysed by SDS gel electrophoresis, and exposed to film before western blotting with indicated antibodies.

Phosphorylation of both CRMP-2 S518A and T514A by p25/Cdk5 was observed (Fig. 5.20 lane 4 and 5) confirming specificity of the two kinases and the general maintenance of protein properties/structure by these mutations.

In vitro phosphorylation by GSK3β appeared to reduce the mobility of CRMP-2 in SDS gels, and phosphorylated bands aligned with the reduced mobility species (Fig. 5.19 lane 2, 3 and 7). To examine whether GSK3β could phosphorylate CRMP-2 *in vivo*, Cos-7 cells were transiently transfected with CRMP-2, GSK3β and p35/Cdk5 cDNAs and cell lysates analysed (Fig. 5.21).

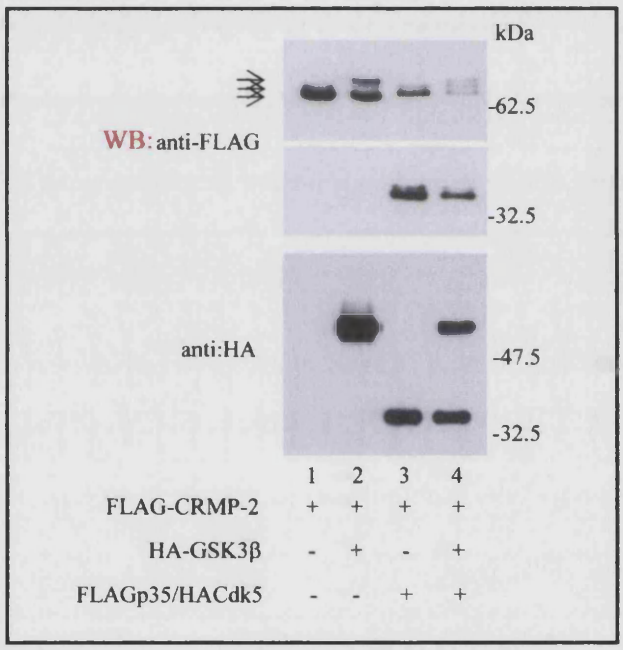

Fig. 5.21 Lysates from Cos-7 cells expressing CRMP-2, GSK3β and p35/Cdk5.
Cos-7 cells were transiently transfected with indicated plasmids and 16 hrs post transfection cell lysates were analysed by 9% acrylamide SDS gel electrophoresis and western blotted with indicated antibodies

From the cell lysates of Cos-7 cells over-expressing FLAG-CRMP-2 alone (Fig. 5.21 lane1), the majority of expressed protein migrates as a single band with a very faint band of reduced motility. However when co-expressed with GSK3β, CRMP-2 appeared as a prominent doublet (Fig. 5.21 lane 2). From the previous *in vitro* kinase

assay (Fig. 5.19) and published results (Gu et al., 2000) it is possible these bands with reduced mobility represent phosphorylated forms of CRMP-2. Interestingly p35/Cdk5 expression, which probably phosphorylates a single site, did not induce a mobility shift in CRMP-2 when co-expressed but led to reduced levels of CRMP-2 making the visualisation of the faint doublet clearer (Fig. 5.21 lane 3). Expression of CRMP-2 with p35/Cdk5 and GSK3β led to a further reduction in CRMP-2 expression levels but with resolution of three clear bands of varying phosphorylation (Fig. 5.21 lane 4).

The effects of the various point mutations on *in vivo* phosphorylation of CRMP-2 by GSK3β, were undertaken by transfecting Cos-7 cells and analysing the mobility of FLAG-CRMP-2. Cdk5 is expressed endogenously in Cos-7 cells, although presumed to be inactive due to the lack of expression of its activators p35/p39.

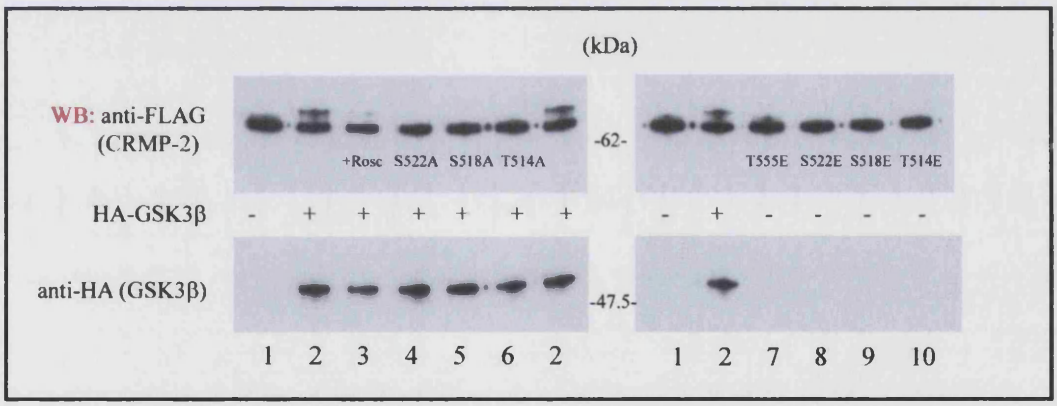

Fig. 5.22 Lysates from Cos-7 cells expressing mutated CRMP-2 proteins with GSK3β. Cos-7 cells were transiently transfected with indicated CRMP2 constructs +/- GSK3β and in one instance treated with 10 μM Roscovitine (+Rosc). 16 hrs post transfection, cell lysates were analysed by 9% acrylamide SDS gel electrophoresis and western blotted with indicated antibodies

Mutations of CRMP-2 S522A, S518A and T514A, all showed reduced *in vitro* phosphorylation by GSK3β, to various extents (Fig. 5.19) and no mobility shift induced by the co-expression of GSK3β in Cos-7 cells (Fig. 5.22 lanes 4, 5 and 6). This implies

phosphorylation of all three sites is required for reduced mobility of CRMP-2, as a presumed result of a conformational change. Mutations of Ser/Thr phosphorylation sites to charged glutamate residue have been used to mimic phosphorylation. However mutations of either CRMP-2 S522E, S518E or T514E had no effect on mobility in SDS gels (Fig. 5.22 lanes 8, 9 and 10). If these mutations do mimic phosphorylation this would argue that a single phosphorylation event at any of these sites is insufficient for a conformational change/mobility shift, but rather a sequential phosphorylation process is required. It is of interest that CRMP-2 T555E also does not induce a conformational change in CRMP-2 (Fig. 5.22 lane 7). Thr^{555} phosphorylation by ROK is implicated in LPA induced growth cone collapse. Phosphorylation of Thr^{555} is apparently not sufficient for conformational change but is required for collapse (Aimura et al., 2000).

The possibility that an endogenous kinases in Cos-7 cells, either Cdk5 or Cdk family member, primes for subsequent GSK3β phosphorylation was investigated using the Cdk inhibitor roscovitine (Fig. 5.22 lane 3). Roscovitine is a powerful inhibitor of Cdk 1, 2, 5, 7 and 9 (Wang et al., 2001) but, unlike many Cdk5 inhibitors, shows minimal activity towards GSK3β *in vitro* (de Azevedo et al., 1997). Treatment with roscovitine did significantly reduce the appearance of the doublet in Cos-7 cells but not completely, possibly implying that endogenous Cdk1,2 5, 7 or 9 and other kinases may phosphorylate Ser^{522} of CRMP-2. The possibility that roscovitine inhibits GSK3-β *in vivo* can also not be entirely ruled out because minimal *in vitro* targets were found to be major *in vivo* targets for the Cdk inhibitor purvalanol (Knockaert et al., 2000).

To further study phosphorylation of CRMP-2 *in vivo* it was sought to investigate the use of phosphospecific antibodies. Cell signalling Technology's Phospho-Thr-Pro antibody has been used to investigate numerous Cdk substrates including Cdk5 phosphorylation of PAK (Rashid et al., 2001). A monoclonal antibody (3F4) had also

been raised against paired helical filaments and the antigen identified as CRMP-2 (Yoshida., et al 1998). It was subsequently realised the antigen represented phosphorylated CRMP-2 at residues Thr^{509}, Ser^{518} and Ser^{522} (Gu et al., 2000). Thus 3F4 represented a good candidate phospho-antibody to study Cdk5 and GSK3β mediated phosphorylation of CRMP-2. Cos-7 cells were transfected with GST-CRMP-2 and combinations of GSK3β, p35 and Cdk5. GST pulldowns were then carried out, analysed by SDS gel electrophoresis and western blotted with these antibodies.

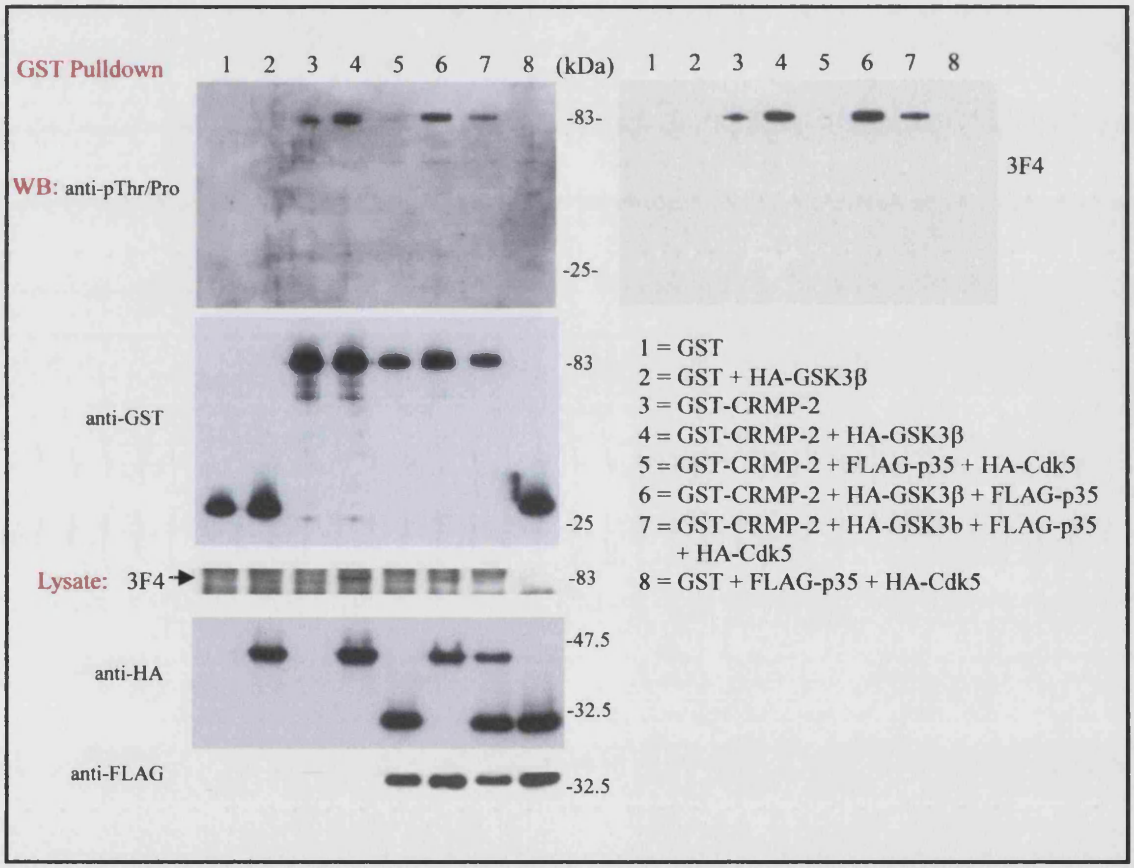

Fig. 5.23 Phosphorylated CRMP-2 detection using phospho-specific antibodies.
Cos-7 cells were transiently transfected with indicated GST-CRMP2 constructs +/- GSK3β +/- FLAG-p35/HA-Cdk5. 16 hrs post transfection, GST pulldowns were carried out and analysed by 9% acrylamide SDS gel electrophoresis and western blotted with indicated antibodies

GST pulldowns from Cos-7 cells over-expressing GST-CRMP-2 plus GSK3β (Fig. 5.23 lane 4) compared with GST-CRMP-2 alone (Fig. 5.23 lane 3) followed by western blotting with phosphospecific antibodies, confirmed increased *in vivo* phosphorylation of CRMP-2 in the presence of GSK3β. Both antibodies recognise this increased phosphorylation. Detection with phosphoThr-Pro antibody is consistent with phosphorylation of Thr-514 by GSK3β (Fig. 5.15) while 3F4 recognition is consistent with phosphorylation of Ser-518 and Ser-522. 3F4 antibody also detects the phosphorylated CRMP-2 in the cell lysates of the over-expressing Cos-7 cells. Both antibodies do react with expressed CRMP-2 alone (Fig.5.23 lane 3) implying CRMP-2 is a target of kinases in Cos-7 cells which is consistent with Gu et al., 2000

Neither antibody could detect any potential increase in phosphorylation of CRMP-2 by p35/Cdk5 (Fig. 5.23 lane5). This is consistent with the specificity of the phosphoThr/Pro antibody. 3F4 recognises phosphorylated CRMP-2 on three residues Thr^{509}, Ser^{518} and Ser^{522} with elimination of any site dramatically reducing affinity. Phosphorylation by GSK3β of two sites (Fig.5.23 lane4) does allow some degree of binding while phosphorylation of Ser 522 alone is probably not sufficient (Fig.5.23 lane 5).

Expression of CRMP-2 with GSK3β plus p35 or p35/Cdk5 did not significantly enhance detection of phosphorylated CRMP-2 (Fig. 5.23 lane 6 + 7) compared with CRMP-2 + GSK3β (lane 4) potentially as a result of reduced expression of CRMP-2.

5.E Summary of Results III

CRMP-2 was identified as an *in vivo* interactor of α2 chimaerin SH2 domain, through immunoprecipitation studies. The model of a closed conformation of α2 chimaerin was also further supported by the lack of interaction between CRMP-2 and α2 chimaerin in the absence of PMA, although a difference in sub-cellular localisation could also account for this. Although the SH2 domain of α2 chimaerin is required for its interaction with CRMP-2, it is still not clear how or whether tyrosine phosphorylation regulates this interaction. Elimination of the ability of α2chimaerin SH2 domain to bind tyrosine phosphorylated residues does not abolish interactions with CRMP-2 in the presence of PMA in an over expressed system. However treatment with pervanadate, enhancing tyrosine phosphorylation of CRMP-2 and α2 chimaerin, enhanced their association only in neuroblastoma cells and not Cos-7 cells. It is also possible α2 chimaerin interacts with multiple or N and C-terminal regions of CRMP-2.

α2 chimaerin was found to associate with numerous phosphotyrosine containing proteins *in vivo* and itself appears a substrate for tyrosine kinases, with Fyn identified as one potential kinase, potentially phosphorylating α2 chimaerin within amino residues 39-196. The amino terminal (1-39) residues were implicated in conformational dependencies.

CRMP-2 did not serve as a substrate for Fyn tyrosine kinase *in vitro* but was identified as a novel substrate for Cdk5 activity *in vitro* and the site of phosphorylation mapped to Ser^{522}. GSK3β was found to phosphorylate CRMP-2, *in vitro* and *in vivo*. GSK3β phosphorylation potentially requires previous phosphorylation of Ser^{522}, possibly by Cdk5, allowing for subsequent GSK3β phosphorylation of Ser^{518} and

Thr^{514}, which together brings about a conformational change in CRMP-2, reducing mobility in SDS gels.

Chapter Six

Discussion

6.A α2 chimaerin

α2 chimaerin consist of three distinct protein domains. A catalytic Rho GAP domain is believed largely specific towards Rac1, with little activity towards Cdc42 and none towards Rho *in vitro* (Diekeman et al., 1991, Manser et al., 1992), although a complete investigation against all the Rho GTPase members, remains to be undertaken. The presence of a C1 domain and a putative SH2 domain also implicate regulatory lipid and protein interactions associated with tyrosine phosphorylation pathways.

6.A.1 Lipid Interactions

Rho GTPases cycle between GTP and GDP bound forms and flip from cytosolic to membrane distributions. It is generally believed Rho GTPases must be GTP bound and associated with membranes to elicit downstream signalling, with prenylation or GDI association determining location. Evidence favours the model of GDI dissociation with subsequent Rho GTPase translocation, followed by GTP exchange on the membrane (Robbe et al., 2003). Numerous regulators of Rho GTPases thus require lipid localisation, with many exchange factors possessing PH domains, for this function.

The C1 domain of the Rac GAP chimaerins is involved in lipid and phorbol ester associations (Ahmed et al., 1990). α1 chimaerin adopts a predominantly insoluble subcellular localisation (Hall et al., 2001). Conversely α2 chimaerin is largely soluble, presumably through influences of its variant N-terminus, encompassing a SH2 domain. A mutation within the SH2 domain, maintaining phosphotyrosine binding, but potentially locally altering charges surrounding the phosphotyrosine pocket (N94H), translocates α2 chimaerin, promoting lipid associations (Hall et al., 2001, Fig.3.1). Phosphotyrosine binding knock out mutations (R56L, R73L) have no effect on α2

chimaerin localisation (Hall et al., 2001), implying phosphotyrosine dependent associations of the SH2 domain are not responsible for the cytosolic location, at least under these over- expressed, non stimulated conditions in Cos-7 cells. In this study, a second mutation of the GAP domain, a deletion of three residues (Δ303-305), was also found to translocate α2 chimaerin, while the point mutation R304G was without any effect on distribution (Fig.3.1). Both these mutations abolish GAP activity but maintain Rac binding (Ahmed et al., 1994) implying the differences are independent of Rac association and GAP activity. The loss of Tyr^{303} through Δ303-305 with subsequent translocation, could implicate an intramolecular interaction between the SH2 domain and Tyr^{303}. Although the critical nature of this residue was not investigated, phosphorylation of this site could potentially serve as a negative regulatory mechanism for α2 chimaerin activity, increasing intramolecular interactions. Alternatively loss of the critical 303-305 may be such a drastic mutation that loss of native conformation is inevitable.

Exogenous expression of α2 chimaerin Δ303-305 or α2 chimaerin N94H in Cos-7 cells revealed a perinuclear punctuate accumulation of these translocated proteins, in contrast to the uniform cytosolic staining of α2 chimaerin (Fig. 3.1). PMA treatment of N1E-115 neuroblastoma cells over-expressing α2 chimaerin also resulted in a clear translocation of this phorbol ester receptor from the cytoplasm to Triton-x-100 soluble and insoluble fractions, representing both membrane and cytoskeletal elements (Fig. 3.2). PKC involvement in this PMA induced translocation was not ruled out but other published results have shown this is PKC independent for the highly related β2 chimaerin (Caloca et al., 1997, 1999, 2001). Exogenous expression in Cos-7 cells and live cell fluorescent imaging also showed a punctate aggregation of GFP-α2 chimaerin

in the cytoplasm concentrating around the nucleus upon PMA treatment (Fig.3.9), with no observable plasma membrane association.

The Golgi complex is the central protein sorting structure of the cell. α1 chimaerin has been shown to regulate Golgi stability during interphase (Alonso et al., 1998) while β2 chimaerin translocation to Golgi as well as plasma membrane in over expressing Cos cells has also been observed upon PMA treatment (Caloca et al., 2001). β2 chimaerin associates with the transmembrane protein Tmp21-I via the C1 domain (Wang et al., 2002). The transgolgi network is rich in DAG, which is required for the recruitment of the vesicle biogenesis factor, PKD (Protein kinase D), via its C1 domain (Baron et al., 2002). The majority of DAG production at the Golgi is believed mediated by two separate pathways. PLD is localised to the Golgi (Freyberg et al., 2001) and converts the abundant phosphatidylcholine (PC) into phosphatidic acid (PA), which is subsequently dephosphorylated by PA phosphatases (PAPs), resulting in DAG production. Alternatively, the Golgi enzyme sphingomyelin (SM) synthesase interconverts PC and SM, which in the production of the vast amount of Golgi SM (Pagano et al., 1988), liberates DAG. Thus various C1 containing proteins, including PKC ε (Lehel et al., 1995) and human Munc13 (Song et al., 1999) may be translocated to Golgi as well as plasma membranes. In the case of RasGRP, the translocating membrane was determined by the concentration of PMA (Lorenzo et al., 2001) while specificity of phorbol ester anologues can also modulate targeting as shown by PKCδ which translocates to plasma or nuclear membranes accordingly (Wang et al., 1999). Thus it is likely that *in vivo* the concentration and specificity of DAG production plays a major role in targeting these phorbol ester receptors.

Until recently, Ras activity was thought restricted to the plasma membrane, but has recently been found active on the Golgi complex (Chiu et al., 2002) through PLCγ production of DAG and recruitment of the C1 containing Ras exchange factor, RasGRP1. Cdc42 activity has also been localised to the Golgi apparatus (Erickson et al., 1996, Musch et al., 2001) along with its GEF, Fgd1 (Estrada et al., 2001) and effector IQGAP (McCallum et al., 1998). Rac activity has so far been largely localised to the plasma membrane, although cross talk does exist between Rac and ARF/Rab family members (Radhakrishna et al., 1999) implicating a role for Rac in membrane trafficking. Several Rac GAPs have also been localised to the perinuclear region including MgcRacGAP (Naud et al., 2003), ARHGAP4 (Foletta et al., 2002) and OCRL1 (Faucherre et al., 2003). Interestingly a large pool of inactive Rac-GDP also exists within the perinuclear region of fibroblast cells (Kraynov et al., 2000). α2 chimaerin may thus serve to regulate Rac signalling on endomembranes, controlling vesicle trafficking and it is tempting to speculate a possible involvement in maintaining a perinuclear Rac-GDP pool in neuronal cells.

α2 chimaerin is not endogenous to Cos-7 cells but is expressed in N1E-115 neuroblastoma cells. Neuroblastoma cells permanently over-expressing α2 chimaerin (α2.10) are flattened adhesive cells which were found to display dynamic peripheral activity, indicative of dynamic Rac signalling (Fig. 3.3). Cell staining revealed a predominantly uniform cytoplasmic distribution of α2 chimaerin extending to the leading edge of peripheral lamellipodiall structures (Fig.3.2B). Although difficult to visualise in live cell imaging, due to dramatic morphological effects on activation, fixed cell staining showed α2 chimaerin redistribution with a punctuate accumulation towards the periphery of the cell, upon PMA treatment. The exact nature of this translocation remains unclear but could imply targeting of translocation is cell specific, potentially

determined by local interacting proteins. Over-expression of the α2 chimaerin Δ303-305 mutant in neuroblastoma cells displayed punctate accumulation along the neurite (Fig. 3.8), which suggests a role in vesicle trafficking along neurites.

In summary, either specific mutations of α2 chimaerin or its association with phorbol ester results in translocation to lipid membranes, implying the native protein may reside in a conformation masking the C1 domain from lipid associations.

6.A.1.1 Lipid association and α2 chimaerin GAP activity

The lack of effect of transiently over-expressed α2 chimaerin in Cos-7 (Fig. .31) and N1E-115 neuroblastoma cells (Fig. 3.8), on cell morphology is of interest. Over-expression of an active Rac GAP, in theory, should down regulate Rac activity with presumed dramatic morphological consequences. N1E-115 neuroblastoma cells deprived of serum undergo differentiation and neuritogenesis in a Rac/Cdc42 dependent manner (Kozma et al., 1997), which is inhibited by the over-expression of α1 chimaerin (Hall et al., 2001) but not α2 chimaerin (Fig. 3.6 and 3.7). This implies the proposed closed conformation of α2 chimaerin inhibits GAP activity as well as lipid associations. Although speculative it is feasible that there is an association of SH2 domain with GAP domain (Tyr^{303}) which could stearically hinder the essential Arg^{304} required for GAP activity. Numerous Rho GAPs appear to reside in an inactive conformation including TCGAP (Chiang et al., 2003) and OPHN-1 (Fauchereau et al., 2003). The N-terminal GTP binding domain of p190RhoGAP has also been found to regulate C-terminal GAP activity (Tatsis et al., 1998) with tyrosine phosphorylation by Src reducing GTP binding (Roof et al., 2000). Thus GAP activity may be transiently and tightly controlled.

Cellular responses to PMA appear varied; Swiss3T3 fibroblasts undergo dramatic ruffling with accompanied macropinocytosis (Ridley et al., 1992), GT1 hypothalamic neurones extend neurites (Choe et al., 2002), human neuroblastoma cells SHSY5Y differentiate (Pahlman et al., 1981) and in DRG and RGC neurones (retinal ganglion cells) neuronal growth cones undergo collapse, with associated endocytosis (Fournier et al., 2000). This is probably in part due to the cell specific or variable expression of different phorbol ester receptors. PKC isozymes were long thought the primary phorbol ester effectors and have been implicated in numerous PMA induced cellular responses, including neurite outgrowth (Choe et al., 2002, Pahlman et al., 1981). The discovery of novel phorbol ester receptors (chimaerins, PKDs, RasGRP, Mun13, DAG kinase γ and MRCK) has meant a re-evaluation of pharmacological studies with these agents. PMA induced fibroblast ruffling may be partially influenced by the C1 containing Ras exchange factor, RasGRP, which is activated by phorbol esters (Lorenzo et al., 2001). Ras activation in turn can lead to Rac activation and lamellipodia formation (see 1.E).

The chimaerins were the first identified non-PKC phorbol ester receptors (Hall et al., 1990). *In-vitro,* lipid association was shown to regulate α chimaerin GAP activity (Ahmed 1993). The acidic phospholipids, phosphatidylserine and phosphatidic acid, were found to up-regulate GAP activity while neutral phospholipids such as phosphatidylcholine and phosphatidylethanolamine were non-regulatory. Numerous other lipids were also found to inhibit GAP activity *in vitro* via C1 association, including LPA, phosphatidylinositol, PI4P and $PI(4,5)P_2$ (Ahmed et al., 1993). Arachidonic acid was the most potent inhibitor of the catalytic domain, but was independent of the C1 domain. Phorbol ester association, which is lipid dependent, does not directly regulate GAP activity *in vitro* although some synergism with

phospholipids is observed in the presence of limited amounts of phospholipids (Ahmed et al., 1993).

Treatment of Cos-7 cells with PMA results in dramatic lamellipodia formation (Fig. 3.10), indicative of increased Rac signalling. This phorbol ester mediated morphological effect was abolished with the exogenous expression of wild type, but not GAP inactive, α2 chimaerin, directly implicating α2 chimaerin GAP activity. High concentrations of PMA, in Cos-7 cells expressing α2 chimaerin, resulted in cellular collapse. Similarly neuroblastoma cells, where α2 chimaerin is endogenously expressed, underwent rapid neurite retraction upon PMA treatment (Fig.3.5). α2.10 neuroblastoma cells also underwent rapid collapse with associated loss of Rac-GTP upon PMA treatment, consistent with α2 chimaerin GAP activation (Fig. 3.3).

From this and previous work it appears α2 chimaerin resides GAP inactive in the cytosol, but is translocated to lipid membranes upon phorbol ester treatment, where its substrate, Rac-GTP, is localised. Lipid association consequently up regulates GAP activity with subsequent down regulation of Rac signalling. The observed collapse of α2.10 neuroblastoma cells and transfected Cos-7 cells expressing α2 chimaerin, occured in the presence of 10% serum where Rho signalling would be prevalent. Thus the removal of the antagonistic active Rac could enhance Rho signalling pathways, leading to actin/myosin contraction. SHSY5Y neuroblastoma cell response to PMA appears regulated by the presence of serum (Muir et al., 1989).

α1 chimaerin is expressed predominantly in the adult brain and is associated with areas of high plasticity (Lim et al., 1992, Hall et al., 2001). α1 chimaerin associates with membranes, inhibiting serum withdrawal induced neuritogenesis of N1E-115 neuroblastoma cells (Hall et al., 2001). Thus *in vivo,* α1 chimaerin does not appear to be regulated by autoinhibition. Regulation must therefore occur through alternative

mechanisms and it is possible protein concentration modulation of α1 chimaerin may be critical.

6.A.2 α2 Chimaerein GAP Activity and Neuronal Morphology

6.A.2.1 Neurites

Neurite retraction of N1E-115 neuroblastoma cells occurred upon PMA treatment. GAP inactive α2 chimaerin mutants, maintaining Rac binding; potentially serve as dominant negative constructs (Ahmed et al., 1994). The results obtained by expression of these mutants implicated a role for α2 chimaerin in this PMA induced neurite retraction, indicative of a novel, non PKC phorbol ester effect (Fig. 3.6). However the use of a PKC kinase inhibitor did not exclude PKC kinase activity in this response. Inhibition of PKC lead to dramatic filopodia formation and neuritogenesis of N1E-115 neuroblastoma cells with no significant collapse upon PMA treatment (Fig. 3.6). PKC inhibition in chick DRG growth cones also induced filopodia extensions while PMA treatment caused filopodia shortening (Bonsall et al., 1999). PMA and PKC kinase activity have been implicated in neuronal growth cone collapse and neurite retraction (Fournier et al., 2000, Zhou et al., 2001, Xiang et al., 2002) but also in neurite outgrowth (Choe et al., 2002). PKCε induces neuritogenesis of neuroblastoma cells independent of kinase activity but dependent on actin binding (Zeidman et al., 1999, Zeidman et al., 2002). PMA treatment induces differentiation and neurite outgrowth of SH-SY5Y neuroblastomas in the presence of serum but also serves to inhibit serum deprived induced neurite outgrowth of these cells (Muir et al., 1989).

The effects of GAP inactive α2 chimaerin constructs implies inhibition of endogenous α2 chimaerin is sufficient to induce neuritogenesis in N1E-115 neuroblastoma cells. In accordance with this, the over-expression of the "active" α2

chimaerin mutant N94H, supported that α2 chimaerin GAP activity serves to inhibit neuritogenesis. However a possible effector/adaptor function of α2 chimaerin in neurite outgrowth, independent of GAP can not be ruled out. Neuritogenesis requires the forces generated from the cytoskeleton to break the neuronal sphere and, in the case of N1E-115 neuroblastomas, is Rac/Cdc42 dependent (Kozma et al., 1997). In primary hippocampal neurones lamellipodia are the observed direct precursors to neurites (Dehmelt et al., 2003). Consequently, promoters of Rac signalling and actin polymerisation such as Tiam, a Rac GEF, induce neuritogenesis (Leeuwen et al., 1997) which α2 chimaerin, through its GAP domain appears to inhibit it.

Over-expression studies can be misleading since there are numerous associated problems, including mislocalisation and possible sequestration of endogenous signalling pathways. Transiently over-expressed α2 chimaerin does not appear to sequester Rac or DAG, as the expressing neuroblastoma cells still appear to respond normally to serum withdrawal. Thus observed effects from expression of GAP inactive α2 chimaerin constructs are presumably through specific competition with endogenous α2 chimaerin for DAG recruitment or Rac-GTP association. siRNA studies would be an alternative approach to examine knock out of α2 chimaerin function in these cells.

Dynamic Rho GTPase activity is required for proper functioning. In theory α2 chimaerin, in coordination with a GEF (eg.Vav/Tiam), could promote Rac cycling and signalling and, in the case of neuroblastoma cells, cause neuritogenesis. This was not observed in transient over-expression studies. However enhanced expression does not necessarily result in enhanced signalling, especially where dynamic activity is required, involving a delicate balance of factors. It is noteworthy that permanent neuroblastoma cells over-expressing α2 chimaerin to a much lower degree, do display increased

neuritogenesis (Hall et al., 2001). In these cells α2 chimaerin is localised to fluctuating lamellipodia, indicative of dynamic Rac signalling (Fig 3.3).

The observed neurite retraction of N1E-115 neuroblastoma cells in response to PMA could implicate a role for α2 chimaerin activity in axonal retraction. Neuritogenesis and elongation require co-ordination between actin and microtubule cytoskeletons and thus proteins which can interact with both cytoskeletons are likely to play major regulatory roles. Microtubules have a known role in neurite stabilisation and evidence for Rho GTPase regulation of microtubules is emerging (1.E.2.5). Rac-GTP binds tubulin *in vitro* (Best et al., 1996) and α2 chimaerin also associates with tubulin via its SH2 domain *in vitro* (Ferrari Thesis 1999) and could therefore regulate Rac function there. The localisation of α2 chimaerin to endomembranes could also implicate a role in vesicle trafficking along neurites, where microtubule based membrane transport is a limiting factor for axonal outgrowth. Axonal extension/retraction is also regulated by a balance of forces generated from actin and microtubule based motor proteins (Gallo et al., 2002). Rho serves to regulate myosin II activity in neurones, promoting retraction. Thus activation of α2 chimaerin at the leading edge of neurites and down regulation of the Rho antagonist Rac, would favour actomyosin induced retraction.

6.A.2.2 Growth cones

F-actin appears to be a major target of extracellular guidance cues. It was thought guidance cues on binding their receptor, and through the recruitment of GEFs/GAPs, would regulate the Rho GTPases and subsequently the actin cytoskeleton, leading to either protrusive or collapse activities (see Fig. 6.1). α2 chimaerin is a neuronal specific Rac GAP which could be recruited to activated receptors either through receptor

tyrosine phosphorylation or localised DAG production. Thus if Rac caused outgrowth, through induced actin polymerisation, antagonised by Rho collapse mediated by actomyosin contraction, it could be theorised α2 chimaerin recruitment and activation would lead to decreased Rac signalling and growth cone collapse through Rho activation.

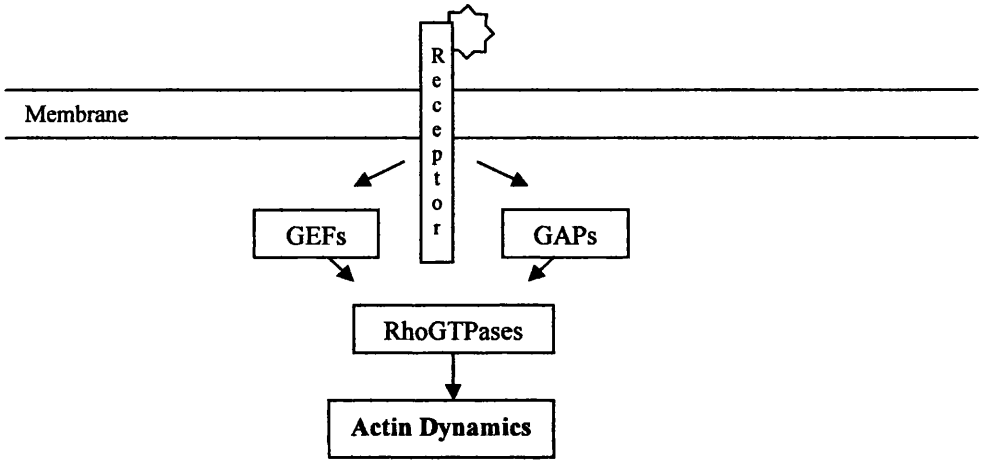

Fig. 6.1 Growth cone guidance and the Rho GTPases

However a paradoxial requirement for Rac in growth cone collapse, in response to some negative guidance cues is now emerging (Jin et al., 1997, Jurney et al., 2002). It appears growth cone collapse requires the co-ordinated activation of several distinct signalling pathways including inhibition of protrusive activity, F-actin depolymerisation and re-organisation, loss of attachment and endocytosis. F-actin depolymerisation is not sufficient for collapse but rather its reorganisation, loss of attachment and possibly endocytosis is (Jurney et al., 2002).

PMA is a powerful growth cone collapsing agent (Fournier et al., 2000) with PKC implicated in downstream signalling (Zhou et al., 2001). PKC activation has been shown to promote microtubule advance in neuronal growth cones (Kabir et al., 2001) and growth cone response to PMA appears regulated by cytosolic cGMP levels (Xiang et al., 2002). Various collapsing factors including PMA, induce loss of actin bundles within the growth cone without depolymerisation of actin (Zhou et al., 2001). In response to PMA and other collapsing agents such as Sema 3A and Ephrin A-2, endocytosis is activated with accompanying reorganisation of F-actin and Rac into distinct vacuole structures (Fournier et al., 2000, Jurney et al., 2002). Measurement of Rac-GTP levels indicated there is an initial reduction in Rac activity with loss of actin polymerisation (1-3min) followed by a recovery (6-12min) with induction of endocytosis and reorganisation of F-actin (Jurney et al., 2002). Rac signalling is not involved in the depolymerisation of F-actin but is required for endocytosis and F-actin reorganisation. This has been put forward to explain the involvement of Rac in growth cone collapse in response to Sema 3A and Ephrin A-2 (Jurney et al., 2002). α2 chimaerin is endogenous to DRGs (Hall et al., 2001) and it is possible that α2 chimaerin plays a role in the initial decrease in Rac-GTP levels involved in cessation of protrusive activity. Inactivation of Rac is not sufficient to cause growth cone collapse (Jin et al., 1997, Kuhn et al., 1999, Jurney et al., 2002) but may allow the dissociation of existing actin polymerisation complexes surrounding Rac, to re-establish a role in driving F-actin reorganisation and endocytosis. Endocytosis is probably required for the internalisation of receptor bound ligand, which α2 chimaerin may regulate through lipid and protein interactions.

$PI(4,5)P_2$ associates with numerous proteins involved in actin polymerisation, such as N-WASP, profilin, gelsolin and CapZ to overall promote polymerisation. Considerable evidence implicates interplay between Rho GTPases and $PI(4,5)P_2$ synthesis pathways in actin cytoskeleton remodelling (see review Sechi et al., 2000). $PI(4,5)P_2$ has been shown to dissociate Rac from Rho GDI (Chuang et al., 1993) and in addition, Rac has been found to associate with numerous enzymes involved in $PI(4,5)P_2$ metabolism. Rac associated with PIP5K *in vitro* (Tolias et al., 1995) and in cells, Rac GTP translocates PIP5K to the cell membrane (Chatah et al., 2001) for localised actin polymerisation through $PI(4,5)P_2$ synthesis (Tolias et al., 2000). Phosphatidic acid (PA), generated from either PLD or DAG kinase activities, is a potent activator of PIP5K. DAG kinase also binds Rac (Tolias et al., 1998) and Rac-GTP has been found to associate with a lipid kinase complex including PIP5K and DAG kinase (Carpenter et al., 1999). Thus Rac may bring PIP5K and PA in close proximity for localised $PI(4,5)P_2$ production. Conversion of $PI(4,5)P_2$ to $PI(3,4,5)P_3$ by PI3K activity may also recruit WAVE2 for lamellipodia formation (Oikawa et al 2004) or Rac-GEFs to maintain signalling. The system is thus set to promote $PI(4,5)P_2$ production and actin polymerisation at the leading edge, with the removal of DAG and the production of chimaerin inhibitors PA, $PI(3,4,5)P_3$ and $PI(4,5)P_2$ (Ahmed et al., 1993). A burst of DAG production could recruit and activate $\alpha 2$ chimaerin down regulating Rac signalling, collapsing the system. This could occur either through the recruitment and activation of PLC, cleaving $PI(4,5)P_2$, or by the actions of PA phosphatases (PAPs). Thus Rac-GTP, promotion of actin polymerisation, via PIP5K activity, can be rapidly quenched with the activation of $\alpha 2$ chimaerin.

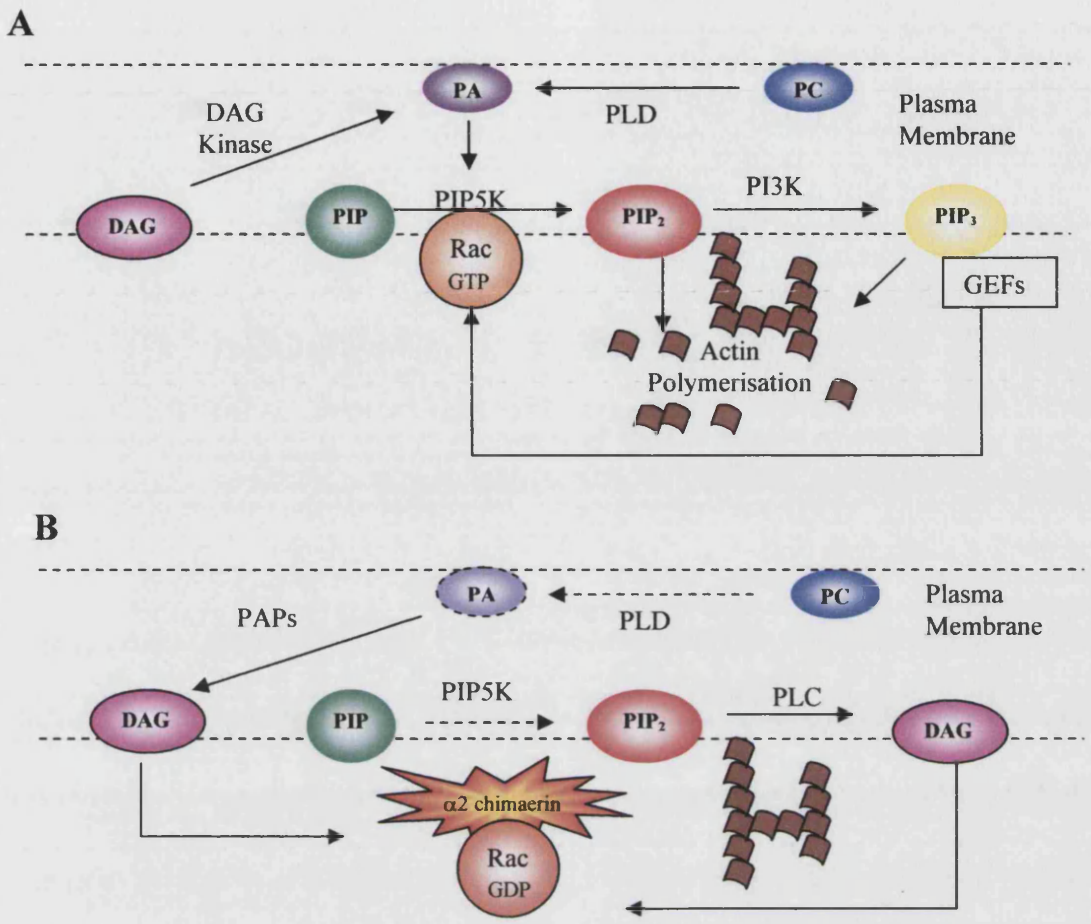

Fig. 6.2 Lipid Metabolism, Rac and Actin Polymerisation. (A) Rac/PIP5K induced PIP_2 synthesis, promotes actin polymerisation and growth cone advance. (B) DAG production via PLC or PAP activities, induces α2 chimaerin GAP activity with subsequent cessation of actin polymerisation.

However, evidence so far appears to be favour PIP5K as a negative regulator of neurite outgrowth, inhibiting neuritogenesis and acting downstream of negative guidance cues including Sema3A, through the destabilisation of focal adhesions possibly via influences on vinculin (van-Horck et al., 2002).

Rac is also directly involved in $PI(4,5)P_2$ depletion through its $PI(4,5)P_2$ phosphatase effector, Synaptojanin 2 (Malecz et al., 2000) and possibly through OCRL1, a bifunctional $PI(4,5)P_2$ 5 phosphatase and RacGAP (Faucherre et al., 2003). Thus dynamic Rac activity is crucial for $PI(4,5)P_2$ turnover, possibly regulating actin polymerisation, endocytosis, vesicle trafficking and focal adhesions, which α2

chimaerin in turn may modulate. α2 chimaerin thus possibly plays an initial regulatory role in neuritogenesis which continues through neurite development as a negative guidance mediator.

6.A.3 Protein Interactions

α2 chimaerin GAP activity causes dramatic morphological effects and consequently must be tightly regulated, which the proposed model of an auto-inhibited conformation would allow. DAG/phorbol ester association induces translocation to lipids with subsequent GAP activation. How is translocation regulated to bring α2 chimaerin in close proximity to the specific membrane at the right location for DAG association and lipid insertion? Selectivity of DAG associations could confer a degree of specificity upon translocation but protein interactions are also likely to be modulatory. In the case of PKC there are three distinct groups of interacting proteins: receptors for activated C kinase (RACKs), substrates and inactive PKC anchoring proteins. It is conceivable the same applies for α2 chimaerin. Rac represents the only identified substrate although others may exist, while the transmembrane Golgi protein Tmp21 serves as a Golgi anchoring protein for β2 chimaerin, through associations with the C1 domain (Wang et al., 2002).

During this study three novel interactors of α2 chimaerin were identified. P35 and Cdk5 were both found to associate with the GAP domain of α2 chimaerin (Fig.4.1, Fig.4.9) while CRMP-2 was identified as a novel SH2 domain interactor in cells (Fig. 5.2). These protein interactions of α2 chimaerin were increased in the presence of phorbol ester. An increase in β2 chimaerin binding to both Rac and Tmp-21 in the presence of phorbol ester has been previously noted (Caloca et al., 2001, Wang et al.,

2002). PMA has been shown to translocate α2 chimaerin and therefore an increase in co-localisation of chimaerin with membranous p35 or CRMP-2 might explain this increased association. Alternatively, a conformational change upon PMA association could unmask protein interacting regions, allowing associations with cytosolic Cdk5 as well as p35, CRMP-2 and lipids. Increased binding in the presence of PMA suggests these interactions only occur with the active form of α2 chimaerin (i.e. analogues to RACKs) and may thus serve to localise GAP activity within the membrane or regulate GAP activity.

6.A.3.1 p35/Cdk5

In this study α2 chimaerin did not serve as a substrate for Cdk5 activity *in vitro* and is therefore an unlikely *in vivo* substrate. However due to conformational importance of α2 chimaerin Cdk5 phosphorylation can not be discounted (Qi et al 2004). Despite associations with p35 and Cdk5 separately, α2 chimaerin could not associate with the active p35/Cdk5 complex, implying competitive binding with much lower affinities of α2 chimaerin for p35 or Cdk5. Only in the presence of high concentrations of PMA could α2 chimaerin associate with p35 in the presence of Cdk5 (Fig. 4.11). Whether this represents a trimetric complex or the pull down of two separate complexes remains unclear. *In vivo* in the presence of DAG, α2 chimaerin could compete with Cdk5 for its activator, thus modulating kinase activity. Results with the inactive kinase mutant Cdk5D144N imply p35 and α2 chimaerin association may be negatively regulated through Cdk5 phosphorylation of p35 (Fig. 4.3). Under the appropriate conditions α2 chimaerin could also conceivably regulate Cdk5 activity through modulation of Rac signalling, as p35/Cdk5 activity towards PAK was increased in the presence of Rac-GTP through unknown mechanisms (Nickolic et al., 1998). α2 chimaerin could also

potentially aid in the translocation of p35 or Cdk5 to the membrane. Protein interactions of p35/Cdk5 have been found to both stimulate (Qu et al., 2002) and inhibit (Ching et al., 2002) kinase activity.

Studies have implicated Cdk5 activity in neurite outgrowth and neuronal migration, while recently, a role in growth cone collapse is emerging with downstream Tau phosphorylation (Nakayama et al., 1999, Sazaki et al., 2002, Cheng et al., 2003). In this study Cdk5 activity was not required for neuritogenesis of N1E-115 neuroblastoma cells, and over-expression of p35/Cdk5 inhibited neuritogenesis, causing rounding up of cells (Fig. 4.19). P35/Cdk5 and α2 chimaerin may thus coordinate activities to inhibit neuroblastoma cell differentiation. Cdk5 activity has been found to regulate cadherin mediated adhesion and has recently been placed upstream of FAK (Xie et al., 2003), while neuroblastoma cells continually expressing α2 chimaerin display increased adhesive properties. α2 chimaerin via its SH2 domain may be recruited to tyrosine phosphorylated cadherin-catenin or integrin complexes, regulating cell adhesion and neuronal migration in coordination with p35/Cdk5. P35/Cdk5 is found inactive in a large multiprotein complex (~600kDa) within the cytosol (Lee et al., 1996) and it is possible α2 chimaerin is also present within this complex. P35/Cdk5 activity has also been localised to the Golgi (Paglini et al., 2001) and implicated in vesicle formation and trafficking, which α2 chimaerin could possibly regulate. Recent evidence has implicated Cdk5 activity in synaptic vesicle endocytosis with the re-phosphorylation of endocytic proteins dynamin1 (Tan et al., 2003), amphiphysin (Tomizawa et al., 2003) and Synaptojanin 1 (Lee et al., 2004).

This study also highlighted a potential novel role for p35 and Cdk5, whose associations with α2 chimaerin C-terminus could regulate GAP activity. Both these proteins interact with the GAP domain of active α2 chimaerin and could possibly

stearically hinder Rac-GTP associations. It was of interest to note the dissociation of α2 chimaerin from membrane fractions in the presence of increasing concentrations of p35 (Fig. 4.17). Although not definitive this result may suggest p35 interaction with α2 chimaerin could inhibit lipid binding of the C1 domain, thus dissociating α2 chimaerin from the membrane. Thus p35/Cdk5 could function upstream and downstream of Rac.

6.A.3.2 CRMP-2

CRMP-2 was identified as a novel SH2 domain interactor of α2 chimaerin. α2 chimaerin was found to interact with both N and C terminal halves of CRMP-2 and residues 1-112 was sufficient for interaction making it difficult to map the site(s) of α2 chimaerin association (Fig. 5.5). This is in accordance with CRMP oligomerisation, where almost intact protein is required for strong associations, with both N- and C-terminal regions implicated (Wang et al., 1997).

Initially it appeared CRMP-2 and α2 chimaerin association was one of the few phosphotyrosine independent SH2 domain interactions, as mutation of α2 chimaerin preventing phosphotyrosine binding did not knock out CRMP-2 association in Cos-7 cells, in the presence of phorbol ester (Fig. 5.6). Furthermore inhibiting tyrosine phosphatases with pervanadate treatment in Cos-7 cells over expressing both CRMP-2 and α2 chimaerin also did not enhance their interaction despite increased tyrosine phosphorylation of CRMP-2 (Fig. 5.7). However, this treatment in N1E-115 neuroblastoma cells did enhance interactions (Fig. 5.8), therefore implying tyrosine phosphorylation may be regulatory in neuronal cells. Of interest was the association with CRMP of a ~p80 tyrosine phosphorylated protein in neuroblastoma cells. CRMP members have been shown to form heterooligomers and the recent identification of N-terminal spice variant (~80kDa) suggests this band could represent a CRMP family

member. Alternatively this may be a tyrosine binding SH2/PTB domain protein. This result does not answer whether the p80 phosphotyrosine protein detected upon pervanadate treatment represents increased binding or increased phosphorylation. CRMP-2 is tyrosine phosphorylated in response to Sema 3A treatment and Fps/Fes has been identified as a potential kinase (Mitsui et al., 2002). This study reveals tyrosine phosphorylation of CRMP-2 may recruit SH2/PTB domain containing proteins, including α2 chimaerin.

CRMP-2 is both cytosolic, membranous and is now known to be associated with dynamic microtubules within axons where it is implicated in growth cone guidance and axonal branching (Yuasa-Kawada et al., 2003). α2 chimaerin through interactions of its SH2 domain has also been found to associate with tubulin (Ferrari Thesis 1999), which Rac also binds *in vitro* (Best et al., 1996). It is thus tempting to speculate a role of α2 chimaerin in coordination with CRMP-2 and Rac to regulate microtubule dynamics in growth cones and neurites. Microtubule and actin cytoskeletal cross talk is essential for proper guidance and outgrowth, where stabilisation of actin filament promotes microtubule advance into the growth cone periphery. CRMP-2 also associates with Numb to mediate endocytosis of the adhesion molecule L1 (Nishimura et al., 2003), which α2 chimaerin may also be involved in since this may be a Rac dependent process. No apparent effect on α2 chimaerin GAP activity was evident upon CRMP-2 association (Fig.4.15 and Fig.4.16). CRMP-2 associates with and inhibits PLD_2 activity which may indirectly regulate α2 chimaerin GAP activity.

6.A.4 Tyrosine Phosphorylation

SH2 domain interactions of α2 chimaerin have been previously investigated *in vitro* (Ferrari Thesis 1999). Only weak binding of full length GST-α2-chimaerin to phosphotyrosine-agarose was demonstrated, to which the isolated GST-α2 SH2 domain bound with considerably increased affinity. This is consistent with the proposed closed conformation of α2 chimaerin. Using a GSTα2 SH2 domain column, tubulin and actin from rat brain extracts, were identified as potential interactors, but this may be an indirect association.

In this study an attempt was made to further investigate the role of the SH2 domain in phosphotyrosine associations in cells. Experiments using pervanadate to inhibit tyrosine phosphatases were carried out, which highlighted the likely tyrosine phosphorylation of α2 chimaerin *in vivo*. (Fig.5 .10). This implies α2 chimaerin exists in a dynamic state of tyrosine phosphorylation/dephosphorylation within cells. Phosphorylation of GAPs has been reported (see 1.E.1.2.2.3). In the case of p190RhoGAP, Src phosphorylation occurs close to the GAP domain (Roof et al., 1998) which recruits p120RasGAP, leading to activation. Ser/Thr GAP phosphorylation has also been demonstrated to alter specificity in the case of MgcRacGAP (Minoshima et al., 2003). Interestingly α2 chimaerin appears to be tyrosine phosphorylated within the SH2 containing N-terminus (1-196) *in vitro* by Fyn (Fig. 5.12). Consistent with a closed conformation model, tyrosine phosphorylation of full length α2 chimaerin by Fyn, or by pervanadate treatment was minimal. Deletion of the N-terminal 38 residues resulted in significantly increased phosphorylation by Fyn, thereby also potentially implicating these residues in native conformational regulation. Thus α2 chimaerin may only be a significant target for tyrosine kinases in its active state, attached to membranes. Tyrosine phosphorylation may serve to recruit further regulatory SH2/PTB

domain containing proteins (adaptor function) or may regulate α2 chimaerin GAP activity or SH2 domain /lipid associations. Fyn has been implicated in FAK and paxillin phosphorylation (Thomas et al., 1995) and α2 chimaerin may thus represent a further downstream target to regulate growth cone adhesion and migration.

As well as observed tyrosine phosphorylation of α2 chimaerin, numerous other associated phosphotyrosine proteins were present upon pervanadate treatment (Fig.5.10 and Fig. 5.11). These may represent associated phosphotyrosine proteins interacting via α2 chimaerin SH2 domain, but may equally represent associated proteins becoming phosphorylated upon treatment or alternatively the recruitment of other SH2/PTP containing proteins to bind tyrosine phosphorylated α2 chimaerin. By which ever mechanism, α2 chimaerin appears to interact with numerous proteins involved in phosphotyrosine signalling pathways.

Numerous SH2 domain proteins are recruited to activated RTKs, where Rac activity is also localised (Ridley et al., 1992). PI3K is directly recruited to RTKs, whose lipid products recruit GEFs such as Sos-1. Sos-1/Grb2 plays a role in RTK mediated activation of Ras but Sos-1 activity can be re-directed towards Rac when in complex with E3b1/Eps8 (Innocenti et al., 2002). The SH2 domain of the GEF, Vav constitutively interacts with the receptor tyrosine kinase Mer, independent of tyrosine phosphorylation. Activation of Mer phosphorylates and releases active Vav (Mahagan et al., 2003). PLCγ is also recruited to numerous RTK via SH2 domain interactions (Mohammadi et al., 1993) but it also requires PI3K products, for PH domain mediated lipid localisation (Falasca et al., 1998), leading to localised DAG production. Thus the SH2 and C1 domain of α2 chimaerin could potentially corporate to efficiently recruit α2 chimaerin to transmembrane tyrosine phosphorylated proteins including RTKs, where Rac activity is localised. It is also possible upon recruitment to activated RTKs,

direct tyrosine phosphorylation of α2 chimaerin could occur which may regulate activity.

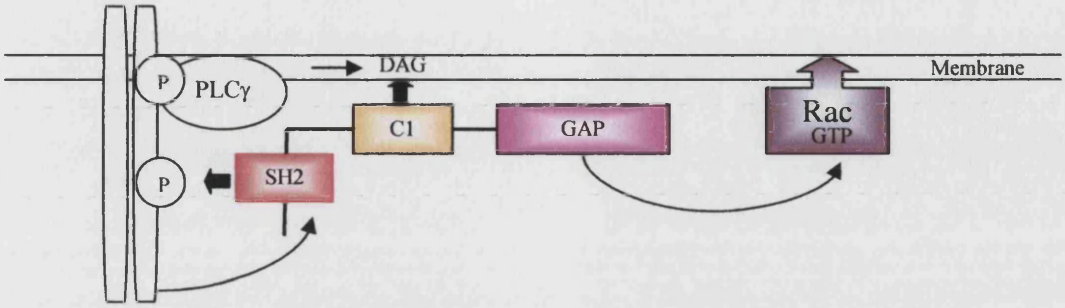

Fig. 6.3 Model of α2 Chimaerin domain cooperativity.

α2 chimaerin SH2 domain and C1 domain cooperate for efficient targeting to phosphotyrosine receptors for localised downregulation of Rac signalling. Receptor tyrosine kinase or associated tyrosine kinases may further regulate α2 chimaerin through tyrosine phosphorylation

6.A.4 Proteolytic Cleavage

During the course of this study it became clear α2 chimaerin is susceptible to proteolytic cleavage by the frequent immunoprecipitation of a N-terminal fragment of α2 chiamerin from cells expression full length protein (Fig. 4.12, Fig.5.11). The detection of an N-terminal ~20kDa fragment potentially implies cleavage could release a ~30kDa C-terminus peptide encompassing the C1 domain and GAP domains, although further proteolysis has not been ruled out. Loss of the SH2 domain through proteolytic cleavage could relieve autoinhibition, releasing the active C1-GAP and may thus be a further regulatory role for this phorbol ester receptor (proteolytic activation). This is reminiscent of PKC regulation, where the hinge region between the catalytic C-domain and the N-terminal regulatory domains is highly sensitive to cellular proteases leading to activation (Ron et al., 1999).

6.B CRMP-2 Phosphorylation

CRMP-2 exists in variable phosphorylated forms *in vivo* (Gu et al., 2000) which is more apparent in the foetal rat brain compared with the adult brain. CRMP-2 has been identified as a target for both ROK (Aimura et al., 2000) and Fps/Fes (Mitsui et al., 2002) kinases. In this study CRMP2 was identified as a novel substrate for both Cdk5 (Fig.5.14) and GSK3β (Fig.5.19) activity *in vitro*. Cdk5 was found to exclusively phosphorylate Ser^{522}, which potentially could prime for GSK3β mediated phosphorylation of Ser^{518} and Thr^{514}. These sites are conserved among some CRMP members (see Fig 5.17) implying other CRMP members may also serve as substrates for these kinases. Phosphorylation of all three sites *in vivo* and *in vitro* was required for a mobility shift in CRMP-2, upon SDS gel electrophoresis, as a presumed result of a conformational change (Fig. 5.22). CRMP-2 phosphorylation could conceivably serve to regulate location or protein interactions. CRMP members form heteroligomers and CRMP-2 associates with Numb, PLD_2, tubulin heterodimers and α2 chimaerin all of which may be regulated by phosphorylation, thus potentially regulating endocytosis, PLD_2 activity, microtubule dynamics and GAP activity respectively.

Cdk5 and GSK3β are two kinases linked to microtubule dynamics. Both kinases phosphorylate Tau and MAP1B (Paglini et al., 1998, Lucas et al., 1998) which have been linked to the stabilisation of microtubules and actin filaments in growth cones (Owen et al., 2003). Adenomatous polyposis coli (APC) phosphorylation by GSK3β reduces its affinity for plus end microtubules (Zumbrunn et al., 2001). In non neuronal cells this signalling pathways is regulated by Cdc42 (Etienne Manneville et al., 2003). CRMP-2 binds preferentially to tubulin heterodimers, rather than polymerised microtubules, promoting polymerisation (Fukata et al., 2002). CRMP-2 thus represents a further substrate implicating p35/Cdk5 and GSK3β in dynamic microtubule regulation

via modulation of both polymerisation and stabilisation. Sequential CRMP-2 phosphorylation also confirms a regulatory relationship between these two kinases as previously demonstrated with Tau (Cho et al., 2003, Hallows et al., 2003).

6.B.1 Sema3A Signalling

Thr^{555} CRMP-2 phosphorylation by ROK is observed in LPA induced growth cone collapse but not Sema3A induced collapse (Aimura et al., 2000). However CRMP-2, p35/Cdk5 and GSK3β have all been implicated in Sema3A signalling (Goshima et al., 1995, Sasaki et al., 2002, Eickholt et al., 2002). In this study potential sequential phosphorylation of CRMP-2 by Cdk5 and GSK3β was observed *in vitro* which could conceivably partly mediate Sema 3A induced growth cone collapse. Cdk5 activity is stimulated in response to Sema 3A, via Tyr^{15} phosphorylation (Sasaki et al., 2002), while GSK3β lies poised inactive at the leading edge of the growth cone, through potential PI3K pathways (Eickholt et al., 2002). PI3K inhibition with resulting GSK3β activation could be PLC mediated, through removal of PI3K substrate $PI(4,5)P_2$ and production of DAG. This could also activate α2 chimaerin. Phosphorylation within CRMP-2 C-terminus, either by ROK or Cdk5/GSK3β, maybe required or sufficient for growth cone collapse. Sequential phosphorylation by two distinct kinases provides a stringent regulatory mechanism. Growth cone collapse can be mediated by both the actin and microtubule cytoskeletons. CRMP-2 binds tubulin heterodimers and thus collapse may be mediated through microtubule regulation or through Numb mediated endocytosis or inhibition of PLD activity (two other associated proteins). Tau is a sequential target of both these kinases which could also mediate growth cone collapse and which is phosphorylated downstream of a number of negative guidance cues including Sema3A (Nakayama et al., 1999, Sazaki et al., 2002, Cheng et al., 2003).

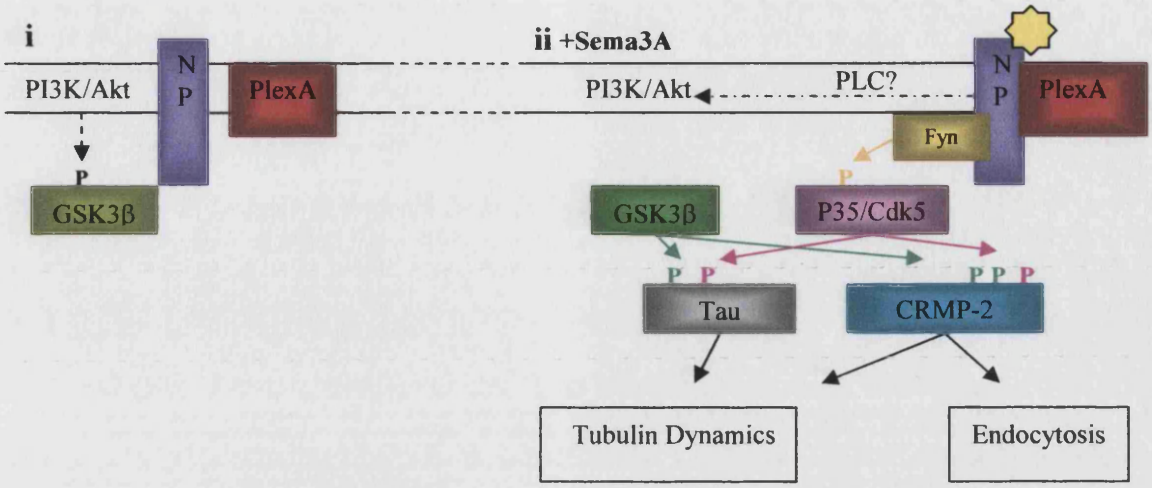

Fig. 6.4 Phosphorylation Pathways Downstream of Sema3A Signalling.
(i)GSK3β is maintained inactive through PI3K/Akt signalling. (ii)Sema3A association with neuropilin (NP) leads to NP/Plex coupling with downstream activation of p35/Cdk5 and phosphorylation of Tau and CRMP-2. This priming event combined with removal of PI3K dependent inhibition of GSK3β allows further phosphorylation of CRMP-2 Ser^{518} and Thr^{514} and Tau, possibly regulating functional interactions and downstream signalling.

Fyn activity has also been implicated in Sema3A signalling (Sasaki et al., 2002) but was shown here incapable of phosphorylating CRMP-2 *in vitro* (Fig.5.12). The CRMP-2 interacting protein, α2 chimaerin, however was phosphorylated by Fyn *in vitro*. Because of its association with numerous Sema3A signalling components (CRMP-2, p35/Cdk5 and Fyn) and its role in neurite retraction, it is conceivable α2 chimaerin is also involved in Sema3A signalling which CRMP-2, p35/Cdk5 or Fyn activities may recruit or regulate. Alternatively tyrosine phosphorylation of the Sema3A receptors PlexA1+2 has been observed (Mitsui et al., 2002, Sasaki et al., 2002) which could recruit α2 chimaerin via SH2 domain interactions. As well as inhibiting actin polymerisation as previously discussed (Fig. 6.1) α2 chimaerin could regulate p35/Cdk5 activity towards PAK, Tau or CRMP-2 with possible subsequent effects on actin/tubulin dynamics and vesicle trafficking during collapse.

6.B.2 Neurodegeneration

Cell signalling involves delicate balances, which if disturbed can be disasterous. Neurodegeneration in some cases appears a disruption of phosphorylation equilibriums, with apparent upregulation of certain kinase activities, leading to hyperphosphorylation of cytoskeletal proteins and neuronal death. Hyperphosphorylation of CRMP-2 on Thr^{509}, Ser^{518} and Ser^{522} has been associated with neofibrillary tangle (NFT) in Alzheimers disease (Yoshida et al., 1998, Gu et al., 2000). As with Tau, Cdk5 and GSK3β activity could be responsible for the majority of this hyperphosphorylation of CRMP-2 in AD (Ser^{518} and Ser^{522}) again implicating these kinases in neurodegeneration. Tau hyperphosphorylation disrupts microtubule associations, leading to microtubule destabilisation. CRMP-2 binds tubulin heterodimers, promoting polymerisation and axonogenesis and it would be of interest to determine how phosphorylation by Cdk5 and GSK3β affect this.

Conclusions

This study has revealed α2 chimaerin to be a tightly regulated molecule probably existing in an autoinhibited state in the cytosol. Activation is achieved through localised DAG production which associates with α2 chimaerin C1 domain, promoting lipid interactions. α2 chimaerin was also found to interact *in vivo* with both p35/Cdk5 and CRMP-2. These protein interactions were enhanced in the presence of phorbol ester and could conceivably serve to target or regulate GAP activity. Tyrosine phosphorylation of α2 chimaerin by Fyn *in vitro* was also demonstrated which is a further likely regulatory mechanism.

α2 chimaerin was shown to be a regulator of neuronal morphology, promoting neurite retraction of N1E-115 neuroblastoma cells. This may implicate α2 chimaerin as

a mediator of negative guidance cues and possibly more specifically Sema3A signalling, where Fyn, CRMP-2 and p35/Cdk5 activities have all been demonstrated.

CRMP-2 was also identified as a substrate for both p35/Cdk5 and GSK3β activities with a possibility of sequential phosphorylation. This sequential phosphorylation mechanism could be a key regulatory point during Sema3A induced growth cone collapse. Hyperphosphorylation of a number of these Cdk5/GSK3β CRMP-2 sites is also associated with NFTs in tissue from Alzhiemers disease patients, further implicating deregulation of both these kinases in neurodegeneration.

Chapter Seven

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